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Vitamin A modulation of iron homeostasis: the role of iron regulatory proteins

by

Stacy Eileen Schroeder

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Nutrition

Program of Study Committee:
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Graduate College

Iowa State University

This is to certify that the master's thesis of

Stacy Eileen Schroeder

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

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LIST OF ABBREVIATIONS

c-acon	cytosolic aconitase
CD163	hemoglobin scavenger receptor
Cp	ceruloplasmin
DcytB	duodenal cytochrome *b*
DMSO	dimethyl sulfoxide (Me_2SO)
DMT1	divalent metal transporter 1
eALAS	erythroid 5-aminolaevulinate synthase
EDTA	ethylenediaminetetraacetate
FPN1	ferroportin 1
H	heavy (ferritin)
HFE	gene encoding a major histocompatibility complex class I-like protein
HPLC	high performance liquid chromatography
IL	interleukin
IRE	iron response element
IRP	iron regulatory protein
L	light (ferritin)
m-acon	mitochondrial aconitase
MHC	major histocompatibility class
mRNA	messenger RNA
MTP1	gene coding for FPN1
PMSF	phenylmethylsulphonylfluoride
RA	all-*trans*-retinoic acid
RAR	retinoic acid receptor
RDA	recommended dietary allowance
RE	reticuloendothelial
ROS	reactive oxygen species
TCA	trichloroacetic acid
TfR	transferrin receptor
TNF	tumor necrosis factor
UL	tolerable upper intake level

ABSTRACT

Iron homeostasis is essential in preventing iron toxicity and deficiency. Several nutrients have been shown to have interactions with iron, specifically vitamin A. A few recently discovered proteins play crucial roles in maintaining iron homeostasis. One central group of governing proteins is known as the iron regulatory proteins (IRPs). Understanding how IRPs respond to altered cellular concentrations of other nutrients may be vital for the treatment or prevention of iron toxicity and deficiency. These studies were conducted to determine if IRPs play a role the perturbation of iron homeostasis in vitamin A deficiency and supplementation. In human hepatoma (HepG2) cells we found that IRP-RNA binding activity was induced when cells were treated with an iron chelator and slightly repressed when administered retinoic acid (RA). Hence, we used a rat model to determine the role of IRPs in iron deficiency, vitamin A deficiency and a combination of these deficiencies. Acute iron deficiency in rats significantly induced hepatic IRP-RNA binding activity and reduced hepatic ferritin to undetectable amounts. Furthermore, we found RA supplementation inhibited the increase in IRP-RNA binding activity to a level not significantly different from the control. This inhibition of IRP-RNA binding activity by RA supplementation was correlated with a 34% reduction in transferrin receptor (TfR) abundance and a partial restoration of hepatic ferritin abundance. Our additional studies utilizing HepG2 cells provided evidence that RA mediates ferritin in a dose-dependant manner. These findings suggest that IRPs play a role in vitamin A mediated alterations of iron homeostasis; providing insight into prevention and treatment of iron deficiency and rationale for cautioning retinoid users of the potential for hepatic iron accumulation.

CHAPTER 1 – GENERAL INTRODUCTION

THESIS ORGANIZATION

The material in this thesis is organized into four main chapters. Chapter one is designed to provide the reader with an organizational overview of the entire document and present the research questions the author addressed throughout the paper. Chapter two is an extensive review of the literature pertaining to the author's research in cellular iron homeostasis and its regulation by iron regulatory proteins and nutrients, specifically vitamin A. A list of references for chapter 2 are provided following the section. The author's research investigating the role of iron regulatory proteins in the perturbation of iron homeostasis by vitamin A status alterations constitutes chapter three. The content of chapter three will be submitted to *The Journal of Nutrition* for publication. General conclusions and ideas for future research paths encompass chapter four. The final portion of the thesis provides a list of the literature referenced throughout chapter 3.

RESEARCH QUESTIONS

Associations between vitamin A status and iron homeostasis have been documented for nearly a century (Koessler *et al.*, 1926). Both human and animal models have been utilized to document the various outcomes of this nutrient-nutrient interaction. **Table 2.1** provides a noteworthy list of human studies that have documented the vitamin A deficiency mediated alterations of iron status indices. Rat studies on vitamin A deficiency have found hampered iron incorporation into erythrocytes (Gardner *et al.*, 1979) and perturbed maternal iron status which causes decreased neonatal iron stores (Andersen *et al.*, 2006). Conversely,

other studies examined the effects of vitamin A supplementation through retinoic acid (RA) administration on iron status indices. Promonocytic U937 cells increased ferritin abundance 3-fold and decreased transferrin receptor (TfR) translation by 70%, but did not affect TfR half-life when administered RA (Iturralde *et al.,* 1992). Vanlandingham and Levenson studied RA administration in neural cells and found that within 24 hours of RA administration brain H-ferritin mRNA increased 4-fold (2003). Despite these numerous novel findings, studies have failed to address the mechanistic relationship between vitamin A and iron.

Recently, iron regulatory proteins (IRPs) have been established as the governing group of proteins that control cellular iron uptake, utilization and storage, e.g. ferritin (Aziz *et al.,* 1987), TfRs (Klausner *et al.,* 1993) and mitochondrial aconitase (Schalinske *et al.,* 1998). This role of IRPs was supported by a study that found rats who consumed an iron deficient diet had a maximal 3- to 5-fold induced IRP activity (Chen *et al.,* 1998). In 2003, IRPs were recognized as orchestrators of the adaptive response to iron deficiency as part of their role in maintaining iron homeostasis (Eisenstein and Ross, 2003). IRPs regulate several iron proteins via posttranscriptional modification of the mRNAs; either stabilizing or inhibiting translation of the target proteins, depending on the site of binding.

Based on the aforementioned research, the goal of our studies was to determine if IRPs play a role in alterations of iron homeostasis during vitamin A deficiency and supplementation. Our hypothesis is that vitamin A and iron deficiencies will decrease ferritin abundance in hepatic tissue which will be mediated by an increase the activity of iron binding proteins. The increase in RNA-binding activity of IRPs will allow them to bind to numerous mRNAs that are responsible for iron homeostasis. This increase in IRP-bound iron

response elements (IREs) on mRNA will consequently decrease iron storage (i.e. ferritin) and increase iron uptake through stabilizing TfR translation. Furthermore, we believe that RA supplementation will reverse these effects by decreasing IRP-RNA binding activity. A decrease in IRP-RNA binding activity will decrease TfR translation and increase ferritin synthesis. These posttranscriptional and translational modifications will in turn increase hepatic intracellular iron concentrations.

CHAPTER 2 – REVIEW OF LITERATURE

IRON HOMEOSTASIS

Iron exists in the environment in several oxidation states, ranging from Fe^{2-} to Fe^{6+}. In the human body however iron is mainly found in the ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms. The ability of iron to change ionic states makes it useful for many biological processes. Heme formation and thus oxygen transport throughout the body, deoxynucleotide synthesis and detoxification are all processes that require iron (Hentze and Kühn, 1996).

Iron is an essential trace mineral along with iodine, selenium and zinc. Iron is the defining trace mineral because it has the highest dietary requirement to the trace mineral defining requirement of less than 100 mg/d. Deficiency of iron results in abnormal physiological and biological functions. Iron is defined as an essential mineral because iron supplementation reverses these adverse functional changes. The importance of iron however extends beyond its current role in the human body. Nobel laureate Christian deDuve hypothesized iron as one of the primary factors in making prebiotic building blocks which ultimately led to the start of primitive life on earth from interaction with ultra-violet light (deDuve, 1991).

Function and Metabolic Usage

Even though iron has a diverse function in the human body, distribution has been categorized by location. Of the 2 to 4 grams of iron in humans, only small amounts are contained in circulation, 1 to 5% in enzymes, 10% in myoglobin, and over 65% in hemoglobin. Daily, humans utilize roughly 80% of the iron requirement to produce

approximately 200 billion new erythrocytes (Beard, 2001). Along with utilization of dietary iron, nearly 20 mg of the metabolic iron requirement is met with iron recycled from senescent red blood cells (Knutson and Wessling-Resnick, 2003). This process is well characterized by Harris *et al.* (1998; **Figure 2.1**). The diagram illustrates the importance of other iron and related proteins in the iron cycle (e.g. Ceruloplasmin; Cp). Cp functions to oxidize ferrous iron for subsequent transferrin binding and delivery to bone marrow. Additionally, the role of transferrin to transport ferric iron and transferrin receptor (TfR) to mediate cellular uptake of transferrin bound iron are illustrated in Figure 2.1.

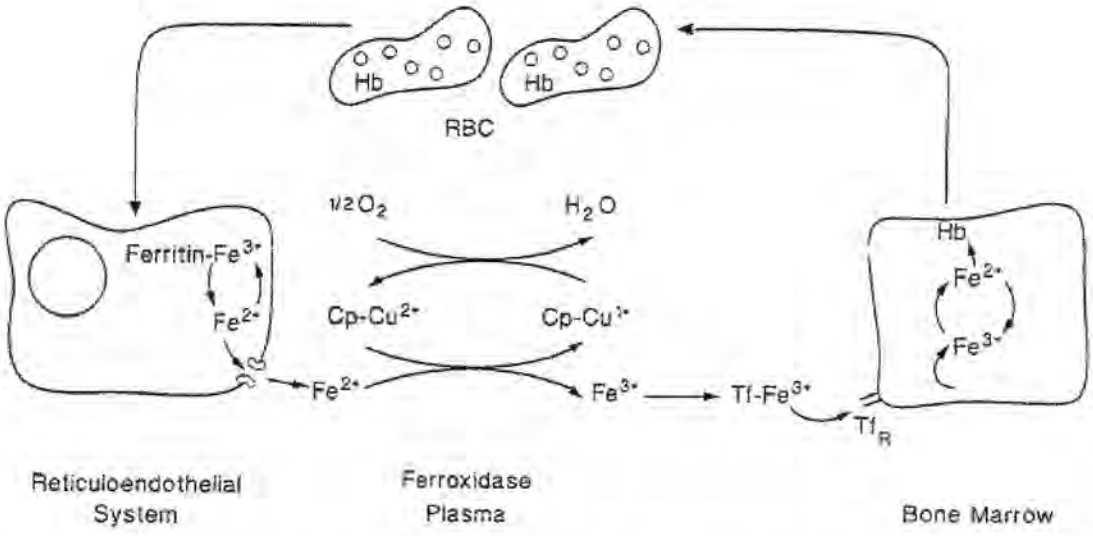

Fig. 2.1. Diagram of iron cycle (Harris *et al.*, 1998). Abbreviations: ceruloplasmin (Cp), hemoglobin (Hb), transferrin (Tf), transferrin receptor (TfR) and red blood cell (RBC).

Even though iron is necessary for many biological processes, accumulation of iron is commonly known to be toxic. This toxicity is explained in part by free iron participating in a redox reaction called the Fenton reaction. This reaction occurs when Fe^{2+} reacts with hydrogen peroxide or lipid peroxides to produce lipid radicals or the most reactive free radical, $^{\bullet}OH$ (Lloyd *et al.*, 1997).

Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$.

The hydroxyl radical has been shown to be directly responsible for cytotoxic effects of oxygen (Bilinski *et al.,* 1985). Furthermore, these radicals have the potential to damage lipid membranes, proteins and nucleic acids (Winterbourn, 1995).

Requirements

The amount of iron utilized daily is 40 mg/kg for women and 50 mg/kg for men (Brittenham and Badman, 2003). These concentrations would be difficult to maintain through dietary consumption. Fortunately, high conservation through recycling of senescent red blood cells is one of the defining characteristics of iron (Knutson and Wessling-Resnick, 2003). Even though the requirements for iron should be fairly distinct because of this recycling process, the recommended dietary allowance (RDA) was admittedly based on a number of assumptions (Yates, 2001). These assumptions were based on the knowledge that body iron content is primarily regulated by iron absorption, which depends on bioavailability. Thus, the calculated human requirement for iron has changed over the years due to characterization of bioavailability in altered iron states (Cook *et al.,* 1974) and iron-nutrient interactions (Apte and Venkatachalam, 1965; Cook *et al.,* 1997; Hallberg *et al.,* 1991). To establish the current requirement a method called factorial modeling was used to factor in basal and menstrual iron losses, and accretion due to fetal development (Food and Nutrition Board, 2000). Consequently, the Food and Nutrition Board (FNB) determined the average loss of iron as 1.3 mg/d in most adult males and 1.7 mg/d in most adult females (Yates, 2001). After adjusting for iron loss amounts and absorption percentages, the FNB established the RDA to range from 7 mg/d for children 1 to 3 years of age up to 27 mg/d for

pregnant women, with requirements at 8 mg/d for adult men and 18 mg/d for pre-menopausal women (Food and Nutrition Board, 2001). The tolerable upper intake level (UL) was determined to be 40 mg/d for infants and children <13 years of age and 45 mg/d for all other age and gender categories.

Additionally, the FNB published a report which stated the recommendations were established on the assumption that 75% of iron intake comes from heme iron sources (Food and Nutrition Board, 2001). Hence, vegetarians who consume their entire iron requirement from non-heme iron, which averages 4-5% iron absorption rather than 10-15% absorption of heme iron, should consume twice RDA value for iron.

Absorption

Divalent metal transporter-1

Divalent metal transporter 1 (DMT1; also known as DCT1 or Nramp2), is an iron transporter located on the luminal side of enterocytes that transports iron into the cell, independent of the transferrin-TfR complex (**Figure 2.2**). Evidence that DMT1 is located on the luminal wall of enterocytes was established when Fleming *et al.* (1997) found reduced iron luminal uptake in an *mk* mouse model defective in DMT1. However, studies using Belgrade rats which have inherited microcytic anemia due to impaired iron transport into immature erythrocytes, have revealed the existence of DMT1 in endosomes for the export of iron (Garrick *et al.*, 1999; Oates and Morgan, 1996). Every cell type investigated has contained DMT1 mRNA, indicating its extensive distribution and highly conserved structure across organisms (Mims and Prchal, 2005).

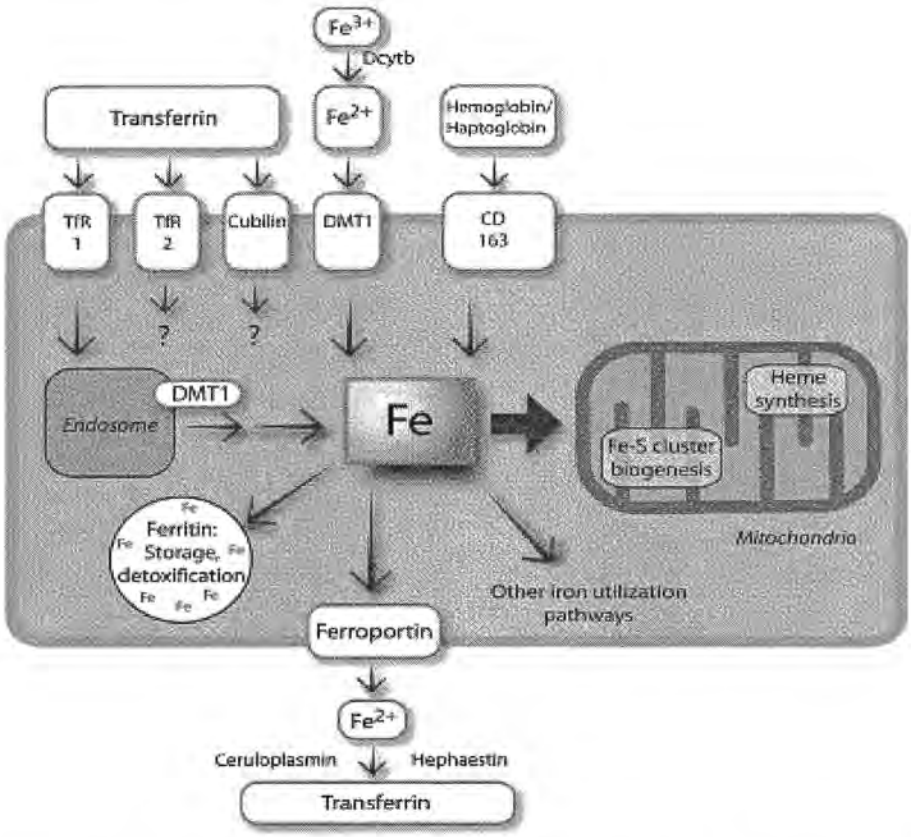

Fig. 2.2. Cellular iron metabolism in a general mammalian cell (Hentze *et al.*, 2004). Abbreviations: divalent metal transporter 1 (DMT1), hemoglobin scavenger receptor (CD163), transferrin receptor 1 (TfR1) and transferrin receptor 2 (TfR2).

DMT1 not only transports iron, but also imports other divalent metal cations, including Fe^{+2}, Zn^{+2}, Mn^{+2}, Co^{+2}, Cd^{+2}, Cu^{+2}, Ni^{+2}, and Pb^{+2} (Aisen *et al.,* 2001). An H^{+} electrochemical potential gradient energizes DMT1 to transport ferrous iron (Makenzie and Garrick, 2005). However, dietary consumption produces luminal iron in the Fe^{+3} state which cannot be utilized by DMT1. Hence, absorption is facilitated by ferric reductases on the brush boarder of the intestine, namely duodenal cytochrome *b* (DcytB; Ma *et al.*, 2006). DcytB and other ferrireductases mediate the reduction of Fe^{+3} to Fe^{+2}, thereby providing the form of iron that can be transported by DMT1 (Mackenzie and Garrick, 2005). One study

has suggested that DcytB may not be necessary for intestinal iron absorption (Gunshin *et al.*, 2005). This study found that the targeted disruption of the *Cybrd1* gene coding for the homolog of DcytB in mice consuming low iron diets did not exhibit a significant change in liver non-heme iron at week 8 of the study. This indicates that DMT1 may utilize different ferric reductases during iron deficiency.

Copper containing ferroxidases: ceruloplasmin and hephaestin

The copper containing plasma protein, Cp, is necessary for the mobilization of iron from enterocytes and the oxidation of ferrous iron for incorporation into transferrin (Brittenham *et al.*, 2000). Cp is an abundant α-glycoprotein in vertebrate organisms that occupies >95% of the plasma copper (Harris *et al.*, 1998). Hepatic iron accumulation in Cp knockout mice (Harris *et al.*, 1999) and in aceruloplasminemic humans (Yu and Wessling-Resnick, 1998) demonstrates the necessity of Cp for absorption and transport of iron. Additionally, aceruloplasminemia leads to iron accumulation in the reticuloendothelial (RE) system and plasma, similar to observations of atransferrinemia and hemochromatosis (Harris *et al.*, 1998).

Although Cp has been characterized as a ferroxidase (Osaki and Johnson, 1969), its activity has been found to vary between species with swine having 10-fold higher ferroxidase activity than rats (Williams *et al.*, 1974). One study examining the ferroxidase activity of Cp, discovered a membrane-bound Cp homologue, called hephaestin, in mice with sex-linked anemia (*sla*; Vulpe *et al.*, 1999). The ferroxidase activity of these copper containing compounds and expression of hephaestin is shown to be lowered in copper-deficient mice suggesting that decreased enterocyte copper content decreases hephaestin synthesis and/or stability (Chen *et al.*, 2006).

Ferroportin

Following the duodenal uptake of iron into an enterocyte, the majority of iron is transported out of the cell to be utilized by other tissues. Ferroportin 1 (FPN1; also known as Ireg1) is a protein that is homologous to the DMT1 family of metal transporters. The gene encoding for FPN1, MPT1, is located on the basolateral membrane of duodenal epithelial cells and RE macrophages in the liver, spleen, and bone marrow system cells (Abboud and Haile, 2000; Donovan *et al.*, 2000; McKie *et al.*, 2000; Knutson *et al.*, 2005). The MPT1 expression locations suggest that FPN1 not only transports iron from enterocytes, but serves as an iron exporter to cells that recycle iron senescent red blood cells. This theory is supported by evidence that FPN1 abundance is induced in cultured macrophages following erythrophagocytosis (Knutson *et al.*, 2003). MTP1 expression in the liver and duodenum has exhibited reciprocal regulation, such that iron deficiency induces duodenal MTP1 expression but decreases hepatic expression (Abboud and Haile, 2000). In contrast, iron toxicity, such as hemochromatosis, may be a result of mutations in FPN1 (Pietrangelo, 2004). Pietrangelo characterized this mutation by showing iron accumulation in liver macrophages or Kupffer cells.

Recent work has linked hepcidin (an antimicrobial peptide described more in depth in the "Hepcidin" subsection) expression with posttranslational regulation of FPN1 expression (Nemeth *et al.*, 2006). Hepcidin administration was shown to cause internalization of FPN1 decrease export of transferrin bound radiolabeled Fe (^{59}Fe) in HEK cells, signifying the importance of this single protein in iron homeostasis (Nemeth *et al.* 2006).

Transport and Cellular Uptake

Intracellular iron can originate from either iron bound transferrin uptake or recycling from hemoglobin. The hemoglobin scavenger receptor (CD163) is responsible for recycling iron from haptoglobin and hemoglobin in monocytes and macrophages (Kristiansen *et al.*, 2001). However most of the iron uptake from hemoglobin is through phagocytosis of senescent red blood cells. An illustration of the former process and other iron absorption and transport mechanisms, discussed in previous and subsequent sections, are portrayed in Figure 2.2.

Transferrin

The serum protein transferrin was characterized in 1947 as a bilobal molecule which transports Fe^{3+} through the circulation (Laurell and Ingelman, 1947). The transferrin-bound iron is non-reactive complex which can be absorbed in the duodenum (Fleming and Bacon, 2005). Previous theories suggested iron is released from transferrin prior to receptor-mediated uptake on the cellular membrane. These theories were rejected in 1969 when radioautographic display of reticulocytes revealed internalized ^{125}I labeled transferrin molecules (Morgan and Appleton, 1969). This study provided evidence that transferrin existed in the internalized iron containing TfR complex. The internalization of iron bound transferrin is crucial as this complex exists as the only known source of iron for hemoglobin synthesis and provides most of the iron for cellular processes (Aisen *et al.*, 1999). Furthermore, erythrocytes employ nearly 80% of the iron transported daily by transferrin (Brittenham *et al.*, 2000). Fortunately, this erythrocyte transportation cycle is capable of utilizing a single transferrin for 100-200 cycles of iron transport (Katz, 1961).

Recently, more studies have characterized the structure of transferrin. Initially, studies characterized the two lobes of transferrin as the N- and C-lobes (Cheng *et al.*, 2004). Each of these lobes has been determined to have one specific ferric binding site (Dhungana *et al.*, 2004). The C-lobe, unlike the N-lobe, has the ability to bind to transferrin receptor 1 (TfR1; Aisen, 2004). Transferrin has the ability to bind iron in the extracellular neutral pH of 7.4 and release iron when engulfed in an endosome at the pH of 5.6 (Dautry-Versat *et al.*, 1983). Moreover, the transferrin lobes interact in a cooperative manner: when the N-lobe releases iron, it stimulates the C-lobe to release the other iron molecule (Hemadi *et al.*, 2006).

The destitute condition of hypotransferrinemic mice emphasizes the overall importance of the transferrin cycle or any situation in which transferrin is unable to bind iron (Trenor *et al.*, 2000). Any hypotransferrinemic state will lead to the rapid removal of ferrous iron from the circulation and its accumulation in liver and other tissues (Craven *et al.*, 1987).

Transferrin receptors

Cellular membrane receptors are necessary for uptake of transferrin bound iron. The transferrin-iron molecule can bind TfR1 to transport iron into the cell (Cheng *et al.*, 2004). TfR1 has been characterized as a homodimer of disulfide subunits (Hu and Aisen, 1978). Of the three subunits in the protein, one 90-760 residue portion is extracellular, the second hydrophobic 62-89 residue resides in the membrane and the third 61 residue portion is located in the intracellular compartment (Aisen, 2004). TfR1 utilizes clathrin-coated pits to scavenge iron bound transferrin molecules which are subsequently endocytosed (Klausner *et al.*, 1983; Daurry-Versat *et al.*, 1983). Transferrin can also be absorbed through other receptor mediated mechanisms: transferrin receptor 2 (TfR2) and cubilin, as shown in Figure

2.2 (Hentze *et al.*, 2004). After endocytosis, the low pH of an endosome causes the transferrin-TfR complex to release iron (Fleming *et al.,* 1998).

Collectively, TfR1 and TfR2 represent one of the first crucial steps in tissue iron absorption, as most iron-bound transferrin enters cells via these receptors. Thus, TfRs have been extensively studied independently and as a collective unit. Collectively, the two receptors have been found to have 45% sequence identity (Zak and Aisen, 2003). Distribution differs between the two as TfR1 is expressed in most cells and TfR2 is only expressed in hepatocytes, duodenal crypt cells and erythroid cells (Kawabata *et al.*, 2005). The specific expression of TfR2 depicts a more explicit role than TfR1. Supporting this theory, studies have found human mutations of TfR2 to cause hemochromatosis (Camaschella *et al.*, 2000).

Nonetheless, TfRs work together in a synergistic manner to regulate iron absorption. Iron chelation by desferrioxamine has been shown to increase both TfRs in various leukemia cells (Basset *et al.*, 1986; Bomford *et al.*, 1986) and erythroblasts (Abe *et al.,* 1992). However, these receptors are regulated by more than just iron status. For example, erythropoietin, a regulator of red blood cell production, has been shown to increase TfR mRNA levels and iron uptake in human K562 and murine erythroleukemic cells by increasing the binding affinity of iron-regulatory protein 1 (IRP1) to the iron response element (IRE) (Weiss *et al.*, 1997b).

Storage

Ferritin

Iron is stored intracellularly in the ferric state by the protein ferritin. This protein is comprised of two subunits: H (heavy or heart) and L (light or liver). These subunits are synthesized from genes on different chromosomes (Worwood *et al.*, 1985), but have >50% identical sequence identity. The H and L chains form a protein shell of 24 subunits that contains a bundle of up to 4500 iron molecules (Aisen *et al.*, 1999). Generally, ferritin is found at high concentrations in tissues which store iron for body needs and exhibit a high L to H ratio; while the majority housekeeping tissues, have low concentrations of ferritin and a low L to H ratio (Cairo *et al.*, 1991). These generalities suggest that L-ferritin has more involvement in storage of large amounts of ferritin while H-ferritin has a greater ability to quickly supply iron to meet cellular needs.

In addition to supplying iron for cellular needs, ferritin functions to provide a protective cellular role by sequestering intracellular iron and converting it to a less reactive state. This role characterizes ferritin as a ferrihydrite (Lawson *et al.*, 1991; Levi *et al.*, 1994). Specifically, a study utilizing spectroscopy methods found the ferroxidase center to be formed by the residues glutamate, tyrosine and histidine (Pereira *et al.*, 1998). When iron is needed, ferritin is degraded and iron is released for availability to other cellular processes. This process is not well established, but current research shows involvement of lysosomes in intestinal Caco-2, H4-II-E-C3 hepatoma and erythroid K562 cells (Kidane *et al.*, 2006).

Because ferritin is the only iron storage protein, it is thought to accurately reflect iron status. Mei *et al.* recently established ferritin, along with hemoglobin, as the most consistent

indicators of iron response to intervention (2005). Hence, ferritin abundance is measured in many intervention studies where subjects have been examined for chronic disease, as chronic disease would induce anemia. The abundance of ferritin is altered by translational regulation of IRPs. This regulation will be described in depth in the subsequent section.

Iron Regulatory Proteins

The ability for mammals to maintain iron homeostasis involves the modulation of proteins that regulate iron uptake, transport and utilization. These proteins are necessary to prevent iron-catalyzed cellular damage while providing enough free iron for the metabolic needs of the organism. The basic molecular framework for regulation of several of the iron proteins has been established as IRPs. The discovery of IRPs was around the same time as the discovery of the RNA-binding site on mRNA: IRE (Aziz and Munro, 1987; Hentze *et al.*, 1987). IRPs are cytoplasmic RNA-binding proteins that work through posttranscriptional and translational modification of IREs located on mRNA (**Figure 3.1**; Eisenstein and Blemings, 1998). IREs are hairpin loop structures that are located on both the 5'- region and 3'- untranslated region of mRNA. The location, as well as amount of IREs on the mRNA, determines the affinity of IRP binding (Eisenstein and Blemings, 1998). Binding that occurs on the 5' region blocks the translation of the mRNA, consequently decreasing the synthesis of the target protein. If the binding occurs on the 3'-untranslated region, the stability of the mRNA message will increase thereby increasing the protein synthesis from that mRNA. IRP activity is inhibited in states of iron abundance and activated in states of iron scarcity (Barton *et al.*, 1990), thus affects all proteins containing IREs.

The list of proteins established to have mRNAs that contain IREs is continuously expanding. Current IRP target proteins are TfR (Klausner *et al.*, 1993), H- and L- ferritin

(Aziz *et al.*, 1987), DMT1 (Hubert and Hentze, 2002), erythroid 5-aminolaevulinate synthase (eALAS; Dandekar *et al.*, 1991), FPN1 (Zoller *et al.*, 2002) mitochondrial aconitase (m-acon; Kim *et al.*, 1996; Schalinske *et al.*, 1998) and succinate dehydrogenase iron protein subunit (Eisenstein, 2000). In addition, studies have suggested that IRP modulates iron homeostasis by regulating ferritin in a translational manner and TfR in a posttranscriptional manner (Schalinske *et al.*, 1998).

Two structurally distinct proteins comprise IRPs: IRP1 and IRP2. Even though human IRPs have a 61% sequence identity and 79% similarity, IRP2 contains a 73 amino acid N-terminal segment that IRP1 does not contain (Thomson *et al.*, 1999). This insertion is believed to be involved in the regulation of IRP2 degradation (Iwai *et al.*, 1995). Hence, IRP2 degradation is altered in response to cellular iron status whereas IRP1 abundance is not affected (Samaniego *et al.*, 1994). Increases in IRP1 activity are not reflective of increased IRP1 abundance due to bifunctional characteristics of this protein. This characteristic allows IRP1 not to be degraded during high intracellular iron concentrations, but to bind an iron-sulfur cluster, [4Fe-4S], hence altering IRP1 activity. While this binding decreases the IRPs' affinity for binding IRE on mRNA, it causes IRP1 to have enzymatic activity as a compound known as aconitase (Chen *et al.*, 1998).

Nonetheless, equivalent amounts of IRP1 and IRP2 have been shown to bind to the multiple IREs on TfR mRNA (Erlizki *et al.*, 2002), establishing equal affinity between IREs and both IRPs. Additionally, the RNA-binding activity of both IRPs is induced by protein kinase C phosphorylation (Eisenstein *et al.*, 1993; Schalinske and Eisenstein, 1996), although other stressors have revealed alternate affects on IRP1 and IRP2. Induction of IRP1 activity can be due to nitric oxide or hydrogen peroxide, in addition to low iron status (Drapier and

Bouton, 1996; Hentze and Kühn, 1996). Although hydrogen peroxide activation of IRP1 is dependant on extracellular signaling events (Pantopoulos *et al.*, 1997), nitric oxide mediated inactivation of aconitase has been studied under electron paramagnetic resonance to show that disassembly of the [4Fe-4S] cluster to a [3Fe-4S] cluster is responsible for aconitase inactivation (Kennedy *et al.*, 1998). While nitric oxide does not show the same effect on IRP2, other non-iron signals may alter activity, e.g. cysteine oxidation (Iwai *et al.*, 1995) and growth (Aisen *et al.*, 1999).

Finally, a study by Chen *et al.* examined the alterations of IRP activity during iron deficiency (1998). They found that rats consuming an iron deficient diet portrayed changes in IRP1 by day 1 and IRP2 by day 2 of treatment. This same lab group found the maximal induction of IRP1 to be 3-fold on day 4 and 5-fold on day 7 for IRP2. These inductions produced a 50% decrease in m-acon and repressed ferritin levels to an undetectable quantity (Chen *et al.*, 1998).

Other Iron Regulation Factors

Regulation of intestinal iron absorption is crucial for iron homeostasis due to the lack of a regulated excretion pathway. Thus, a molecular component must be available to communicate iron status in other organ systems to the intestine. Evidence that a molecular component exists is portrayed in the body's ability to increase intestinal absorption during states of reduced iron stores or increased iron needs, e.g. an increase in erythropoiesis.

Hepcidin

Recently, an antimicrobial peptide has been recognized that provides the intestinal communication from other tissues. This peptide, known as hepcidin, was named after

hepatic bacteri**cid**al prote**in** and is also called HAMP or LEAP. Hepcidin was first discovered and isolated from human blood and urine (Krause *et al.*, 2000). Genetically, the single human gene has three exons and two introns that code for an 83 amino acid peptide that may be shortened to varying lengths: 20, 22 and 25 amino acids (Park *et al.*, 2001, Pigeon *et al.*, 2001). The smaller peptides are the ones found in urine and the largest peptide is shown as the only form to produce iron deficiency when injected in rats (Rivera *et al.*, 2005).

Hepcidin has been recognized as having a parallel relationship with iron status (Pigeon *et al.*, 2001). Mutations of the hepcidin gene are present in > 80% of hemochromatosis patients (Feder *et al.*, 1996), indicating the importance of hepcidin in regulation of iron homeostasis. Additionally, the liver has been found to produce hepcidin (Park *et al.*, 2001); supporting theories the liver regulates proteins involved with iron absorption. The proposed main cellular targets of hepcidin are the villus of enterocytes, crypt cells, reticuloendothelial (RE) macrophages and hepatocytes (**Figure 2.3**). While there are several proposed mechanisms of hepcidin regulation of iron utilization, the first portion of the diagram below (Figure 2.3A) proposes that hepatic hepcidin synthesis and secretion are stimulated through hepatic TfR2 uptake of transferrin-bound iron (Fleming *et al.*, 2002; Nicolas *et al.*, 2001). This same diagram proposes hepcidin stimulates HFE-TfR1 mediated basolateral iron uptake in duodenal crypt cells. The iron absorption and subsequent intracellular concentration of a crypt cell dictates the amount of iron absorption proteins in the cell once it travels down the villus and becomes an enterocyte. Hence, the crypt cells that have increased iron absorption will mature into villus enterocytes that have decreased iron absorption ability. Additionally, this diagram suggests that hepcidin released from the liver

may increase iron uptake by the HFE-TfR1 complex on RE macrophages, consequently increasing RE macrophage iron retention and decreasing extracellular iron availability to other tissues (Nicolas *et al.*, 2001).

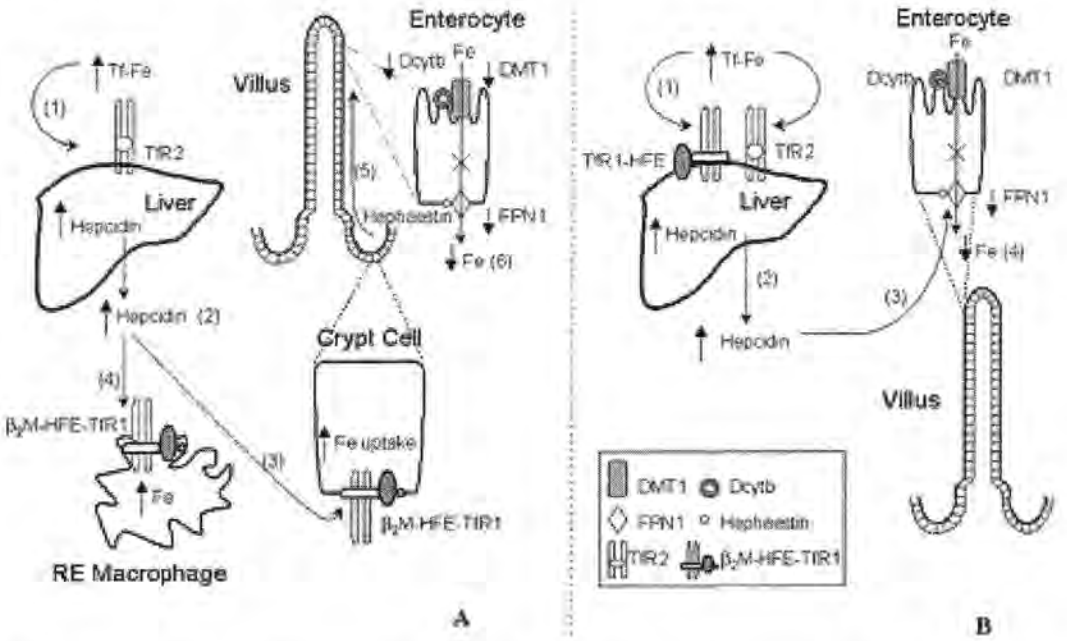

Fig. 2.3. Two proposed theories depicting the role of hepcidin in iron homeostasis (Leong and Lonnerdal, 2004). Abbreviations: divalent metal transporter 1 (DMT1), ferroportin (FPN1), transferrin (Tf) and transferrin receptor (TfR).

An alternative model of hepcidin regulation (Figure 2.3B) proposes that hepcidin synthesis and secretion are altered by hepatic transferrin-bound iron uptake by TfR2 and HFE-TfR1 (Frazer and Anderson, 2003). This model suggests that the increased hepcidin concentration will modulate intestinal iron absorption by altering FPN1 expression in the villous enterocyte, as opposed to the crypt cell (Figure 2.3A) suggested by Nicolas *et al.* (2001). Cellular efflux of iron would be decreased due to hepcidin directly binding to FPN1 thereby inducing its internalization and degradation.

Novel research continues to emerge on this newly discovered peptide. Recent research has sought to better understand the structure-function relationship of this peptide.

One group characterized the N-terminal region of hepcidin-25 as a metal-binding site (Melino *et al.*, 2005). Additional studies indicate that hepcidin-20 exists as a monomer in solution, whereas hepcidin-25 readily aggregates, an idea that supports the existence of different functions of the two peptides (Hunter *et al.*, 2002).

HFE

Although the HFE protein does not bind directly to iron, it does play a large role through association with important proteins involved in iron metabolism. Administering HFE in HeLa (cervical cancer) cells and HEK293 (human embryonic kidney) cells has been shown to decrease iron levels in these cell lines, indicating of the effects of HFE (Enns, 2006). HFE has the ability to associate with a subunit of all major histocompatibility class (MHC) 1 molecules, i.e. β2-microglobulins (the HFE association to TfR1 is reflected in Figure 2.3). This association was established when the HFE deficiency characteristics were shown to be consistent with the iron overload characteristics found in a β2-microglobulin knockout mouse (Feder *et al.*, 1996). This same study analyzed the levels of HFE mRNA in different tissues. Results determined HFE is abundant in the small intestine, liver, pancreas, placenta, kidney and ovary, with low concentrations in the colon, leukocytes, brain and lung (Feder *et al.*, 1996). These results correlate HFE with hemochromatosis because iron accumulation occurs in tissues abundant in HFE (which is discussed further in the "Hemochromatosis" section).

DISRUPTIONS OF IRON HOMEOSTASIS

Deficiency

The first documentation of disease due to iron deficiency (chlorosis) was in 1895 (Guggenheim, 1995). This discovery alerted the medical field and generated more research aimed at examining the characteristics of iron deficiency. Research has shown the necessity of careful orchestration of the proteins involved in iron homeostasis to maintain sufficient and non-toxic iron concentrations. Perturbation of iron absorption, transport or utilization potentially causes iron deficiency in a specific tissue or the whole body. For example, a consistently documented effect of iron deficiency in pregnant women is the causal effect of iron deficiency and preterm deliveries (Zhou *et al.*, 1998; Scanlon, 2000). Moreover, in rats, iron deficiency has not only exhibited adverse consequences on the maternal animal but also perturbs growth and development of the offspring (Andersen *et al.*, 2006).

Iron-deficiency anemia

Iron-deficiency anemia develops when tissues become deficient of oxygen because the lack of iron stifles the production of red blood cells. The lack of normal production of red blood cells primarily affects maximal oxygen consumption. Thus, iron deficiency in muscle cells which decreases oxygen will markedly impair endurance exercise (Willis *et al.*, 1988). There is a difference however between anemia and tissue iron deficiency, as they cause independent effects on various tissues (e.g. skeletal muscle; Davies *et al.*, 1984). The effect of anemia on cardiac muscle has been studied in large populations of >14,000 subjects, which have established anemia as an independent risk factor for cardiovascular disease (Sarnak *et al.*, 2002) and stroke (Schoenberg *et al.*, 1978). Chronic anemia may induce

cardiovascular disease and stroke because of increased cardiac output due to decreased afterload, increased preload and increased chronotropic and inotropic effects (Sarnak *et al.*, 2002).

Nonetheless, anemia is not only caused by insufficient dietary iron intakes. Genetic defects in red blood cells (i.e. sickle cell anemia), hemoglobin molecules (thalassaemia) or bone marrow (sideroblastic anemia) can also cause anemia. Additionally, confounding factors such as cytokines, autoimmune diseases or parasites can produce anemia. A few of these factors will be highlighted in subsequent sections.

Illness and cytokines

The association of anemia and illness dates back to 1932 with the observation of the association of chronic infection and hypoferremia (Locke *et al.*, 1932). Throughout the years, this association has evolved into the understanding that inflammatory cytokines alter the expression of iron transport and storage proteins. However, until recently, there was no explanation for how the expression of these proteins accounted for the irregularity of iron homeostasis, which is seen in the anemia of inflammation.

One theory posited by Weiss *et al.* (1997a) is that interleukin (IL)-4 and IL-13 regulate the iron metabolism of activated macrophages by either opposing the IRP activation by nitric oxide, which induce ferritin translation, or through an IRP-independent increase in TfR mRNA expression. An additional study has reported that repeated administration of IL-6 in rats and cancer patients causes anemia (Nieken *et al.*, 1995). Since this study in 1995, more studies have investigated whether hepcidin plays a role in the anemia of inflammation. A supporting factor in this theory is that inflammatory disease increases macrophage

elaboration of IL-6 which regulates hepcidin production by acting on hepatocytes to increase production of the peptide (Deicher and Horl, 2006). **Figure 2.4** depicts the proposed interaction of inflammation, IL-6 production, hepcidin and iron homeostasis (Andrews, 2004).

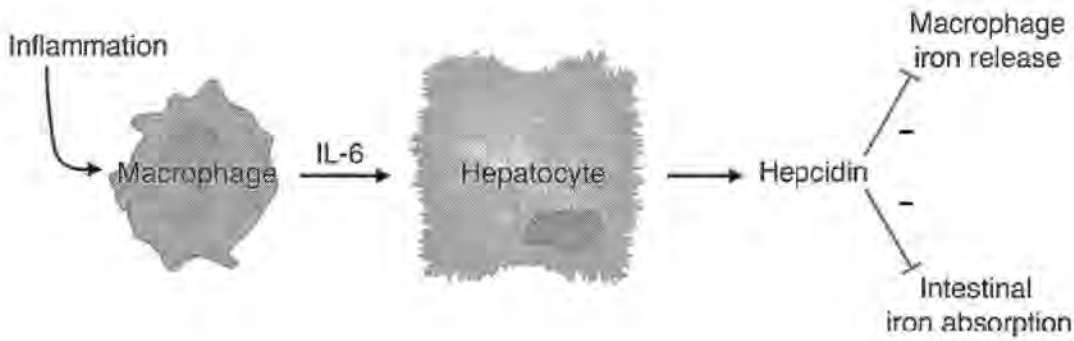

Fig. 2.4. Role of inflammation in iron regulation (Andrews, 2004).

A recent study by Nicholas *et al.* reflected the role of hepcidin in the state of inflammation (2002). Abscesses were induced in mice to model an inflammatory state, which generated a 6-fold increase in hepcidin mRNA content in the liver and a 2-fold reduction of serum iron. This finding was further supported by inducing inflammation in hepcidin-deficient mice (Nicholas *et al.*, 2002). In this model, the lack of hepcidin completely blunted the hyposideremic effect of inflammation, establishing a clear need for hepcidin in the regulation of iron homeostasis during an inflammatory state. A supporting study by Nemeth *et al.* demonstrated that hepcidin is induced specifically by IL-6, not IL-1 or tumor necrosis factor (TNF)-alpha, in human hepatocyte culture, indicating that induction of inflammation from hepcidin is a type II acute-phase response (2003). Furthermore, Nemeth investigated the role of IL-6 in the induction of hepcidin and hypoferremia during inflammation by using IL-6 knockout mice that lack production of hepcidin. Inducing these

mice with inflammation produced an increased hepcidin mRNA expression and decreased serum iron concentration, indicating hepcidin expression is not dependent on IL-6 (Nemeth *et al.*, 2004). As for humans, IL-6 infusion significantly decreased serum iron and transferrin saturation (Nemeth *et al.,* 2004).

Subsequent studies that have cited this inflammation-hepcidin relationship as an established theory have gone on to explore other relationships of IL-6 and hepcidin: time-course studies of inflammatory initiation and hepcidin response (Kemna *et al.*, 2005) and investigation of endogenous expression of hepcidin by macrophages and neutrophils *in vitro* and *in vivo (*Peyssonnaux *et al.*, 2006). Thus, hepcidin has been established as a key regulator in the pathogenesis of the anemia of inflammation.

Toxicity

Variations in iron homeostasis can cause iron to become toxic to cells. The interconversion of iron oxidation states is a mechanism where iron participates in electron transfer, as well as a mechanism whereby iron can reversibly bind ligands. The common biological ligands for iron are oxygen, nitrogen and sulfur atoms (Food and Nutrition Board, 2000).

There are several causes for iron toxicity. Even though dietary iron overload may account for numerous iron toxicity cases, the genetic causes of iron toxicity cannot be prevented as dietary causes. A few genetic causes include: aceruloplasminaemia, congenital atransferrinaemia and hemochromatosis. Hemochromatosis is the most common genetic iron toxicity disease, thus I will focus the next section on numerous causes of this complex disease.

Hemochromatosis

This disease is characterized by a hereditary mutation in the HFE gene coding for a protein that is responsible for moderating transferrin-TfR mediated iron uptake (Roy *et al.*, 1999). The mutated HFE protein fails to associate with the β2-microglobulin and is not transported to the plasma membrane to attach to TfR (Feder *et al.*, 1997). However, this gene does not account for all of the genetic hemochromatosis. Several genes are responsible for causing this disease, many of which have been characterized: HFE gene (the most prevalent mutation), TfR2, hemojuvelin (the cause of juvenile hemochromatosis; HFE2) and hepcidin (Pietrangelo, 2006). Mutations of hemojuvelin produce iron overload due to hemojuvelin's role as a modulator of hepcidin expression (Papanikolaou *et al.*, 2004) and TfR2 has the potential to contribute to iron overload due to its lack of IRE in the 3'-untranslated region, thus lack of regulation by IRPs.

Regardless of the cause, this disease results in iron overload in the plasma compartment and subsequent parenchymal cells, reflected by increased serum transferrin saturation and increasing serum ferritin, respectively (Pietrangelo, 2004). These outcomes have the potential to cause organ damage, disease and unimpaired erythropoiesis. However, diagnosis of hemochromatosis is difficult due to gradual iron accumulation which does not show symptoms until middle age or later (Enns, 2006). Hence, research is constantly developing new and more efficient ways of testing for iron toxicity. For example, this year a research group came out with a novel method utilizing molecular beacon technology to test for mutations in HFE that cause hemochromatosis (Alsmadi *et al.*, 2006).

Oxidative stress

Redox cycling is a characteristic of iron and other transition metals. Reactive oxygen species (ROS) may be produced when the balance of these metals is not perfectly maintained. ROS in any biological system induce oxidative stress. Production of ROS from iron occurs via the Fenton reaction, when hydrogen peroxide oxidizes ferrous iron thereby producing free hydroxyl radicals from iron (Halliwell and Gutteridge, 1984). The Fenton reaction is as follows: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$. Once the hydroxyl radical is produced, it can affect several systems. **Figure 2.5** depicts how lipid peroxidation and DNA damage from the production of iron mediated oxidative stress can lead to the development of serious consequences, and potentially diseases such as cancer.

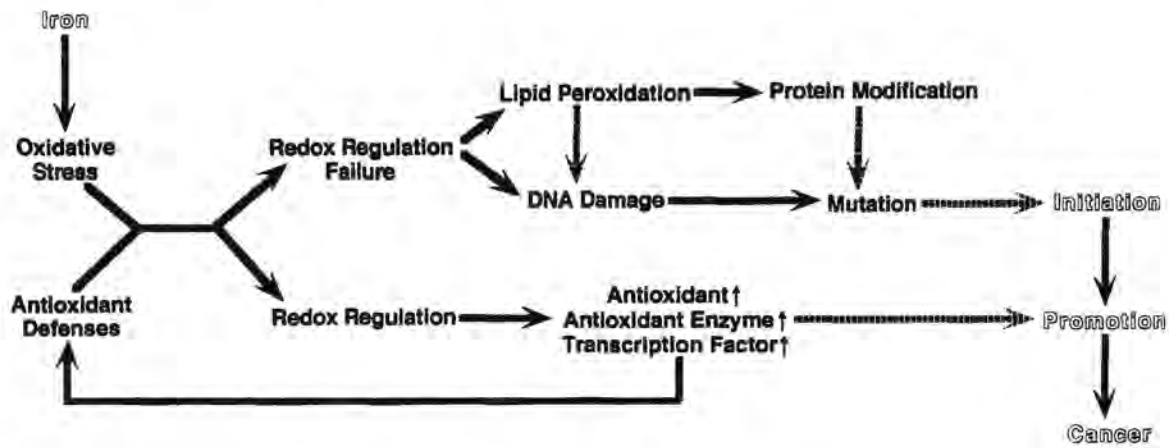

Fig. 2.5. Hypothetical scheme for iron-induced carcinogenesis (Toyokuni, 1996).

The hypothetical scheme for iron-induced carcinogenesis is supported by many factors. Two significant theories in support of the scheme it are that (1) iron can directly produce ROS which induce DNA mutations and (2) iron is an essential nutrient, therefore may help malignant tissues thrive (Stevens *et al.*, 1986).

Interactions with Other Nutrients

Interactions of iron with other nutrients can significantly alter the bioavailability of iron itself or of other nutrients. The amount of iron absorbed has a significant impact on the whole body pool because the utilizable iron pool in humans is determined by two primary factors: the amount of iron absorbed by the intestine and iron supplied from storage sites, e.g. macrophages and hepatocytes. The three iron-nutrient interactions that I will discuss are copper, ascorbic acid and vitamin A.

Copper

Studies have shown that adequate copper stores are necessary for hepatic efflux of iron in rats (Marston and Allen, 1967; Fields *et al.*, 1997). Consequently, hepatocytes accumulate iron during copper deficiency (Reeves and Demars, 2006). Furthermore, a few studies have suggested that iron absorption is impaired in copper deficiency due to decreased levels of total liver iron (Gubler *et al.*, 1952; Lee *et al.*, 1968). In addition to previous findings, Lee *et al.* (1968) found ferritin accumulation in enterocytes, suggesting a problem with mucosal export rather than import.

Recent studies have confirmed that changes in iron absorption are independent of copper levels. Cp knockout mice (Yu and Wessling-Resnick, 1998) and caco-2 cell lines (Tennant *et al.*, 2002; Zerounian and Linder, 2002) did not exhibit alterations of iron absorption due to copper deficiency. However, a study by Chen *et al.* (2006) found inductions of enterocyte FPN1 mRNA and FPN1 protein levels in copper deficient mice of 2.5- and 10-fold, respectively. This study indicates copper deficiency can cause a systemic iron deficiency due to lack of hephaestin. However, copper is not the only nutrient

established to alter iron bioavailability. Another nutrient determined to have a nutrient-nutrient interaction with iron is ascorbic acid.

Ascorbic acid

Observations of iron absorption alterations due to ascorbic acid have been documented for over 50 years (Moore and Dubach, 1951). Numerous studies have established that the increase in iron absorption is due consumption of vitamin C is attributed to increased non-heme absorption and not heme absorption (Apte and Venkatachalam, 1965; Cook *et al.*, 1972; Sayers *et al.*, 1973). Dose-dependent effects of ascorbic acid range from 1.7 to 9.6 times increased iron absorption when ascorbic acid consumption is increased from 25 to 1000 mg (Cook and Monsen, 1977). However, these enhancing effects are eliminated in the presence of an iron absorption inhibitor, such as tea (Disler *et al.*, 1975).

Ascorbic acid utilizes various molecular alterations to promote the uptake of iron. Ascorbic acid not only enhances iron-induced translation of ferritin through decreasing IRP1 activity (Toth and Bridges, 1995), but also mobilizes iron from ferritin by reducing Fe^{3+} to Fe^{2+} (Bienfait and Van Del Briel, 1980). Nonetheless, even though there are several important iron-nutrient interactions, the main nutrient interaction with iron I will focus on is iron and vitamin A.

Vitamin A

Dating back to the mid-19th century, anemia was commonly treated with a potent source of vitamin A: cod-liver oil (Thompson, 1855). Anemia produced by vitamin A deficiency has been established for nearly a century (Koessler *et al.*, 1926). A descriptive

table produced by Semba and Bloem (**Table 2.1**) reflects the vast number of human studies completed in this field in the last 20 years (2002).

Table 2.1 illustrates the importance of vitamin A status on iron homeostasis. Other studies have used animal models to examine the role of vitamin A deficiency in anemia. O'Toole published a study showing that vitamin A deficient monkeys develop anemia, which is correctable with vitamin A treatment (O'Toole *et al.*, 1974). Several groups have found that anemic rats have reduced plasma retinol concentrations and vitamin A accumulation in the liver (Amine *et al.*, 1970; Staab *et al.*, 1984), while the opposite effect occurs in vitamin A deficient rats: hepatic iron accumulation (Sijtsma *et al.*, 1993; Staab *et al.*, 1984; Strube *et al.*, 2002).

Subjects	*Year*[a]	*Location*		*Findings*	*Reference*
Children, preschool	1986	Brazil	52	32.7% with serum retinol < 0.70 µmol/l; 44.6% with hemoglobin < 113 g/l	Araújo et al (1986)
Children, 6–60 months	1993	Pakistan	532	49% with serum retinol < 0.70 µmol/l; 67% with anemia	Molla et al (1993a)
Children	1993	Ethiopia	240	30.2% with serum retinol < 0.35 µmol/l; depressed indicators of iron status	Wolde-Gebriel et al (1993)
Children, 36–83 months	1989	Micronesia	60	Of 31 children with abnormal CIC, 52.9% had hematocrit < 34%	Lloyd-Puryear et al (1989)
Children, 36–83 months	1991	Chuuk[b]	455	23.6% with xerophthalmia; 40% with anemia	Lloyd-Puryear et al (1991)
Children, preschool	1996	Brazil	563	15% with serum retinol < 0.35 µmol/l; 22.2% with anemia	Santos et al (1996); Assis et al (1997)
Children, 4–24 months	1999	South Africa	115	37% with serum retinol < 0.70 µmol/l; 63% with anemia	Faber & Benade (1999)
Children, 18–36 months	2000	Mexico	219	29% with serum retinol < 0.70 µmol/l; 70% with hemoglobin < 115 g/l	Allen et al (2000)
Children, 1–5y	2000	Honduras	1243	14.2% with serum retinol < 0.70 mol/l; 29% anemic; 15.5% w/both	Albalak et al (2000)
School-aged children	1999	South Africa	131	51% with serum retinol < 0.70 µmol/l; 22% with anemia	Oelofse et al (1999)
School-aged children	2000	Bangladesh	164	20% with serum retinol < 0.70 mol/l; 31% with anemia	Persson et al (2000)
Girls, 10–19y	1998	Malawi	118	27% with serum retinol < 0.70 µmol/l;11% with anemia	Fazio-Tirrozzo et al (1998)
Boys, girls, 10–17y	2000	Bangladesh	861	2.1% with xerophthalmia; 94% of males, 98% of females with anemia	Shahabuddin et al (2000)
Pregnant women	2000	Malawi	697	88.8 with serum retinol < 1.05 µmol/l; 73.1% with anemia; all HIV positive	Semba et al (2000a)
Pregnant women	2000	Nepal	390	20% with serum retinol < 0.70 µmol/l; 78.9% with anemia	Bondevik et al (2000)
Pregnant women	2000	Nepal	336	54.2% with serum retinol < 1.05 µmol/l; 72.6% with anemia	Dreyfuss et al (2000)

Table 2.1. Summary of characteristics of epidemiological studies on vitamin A deficiency and anemia completed from 1985 – 2000 (Semba and Bloem, 2002).

Studies on iron absorption vary depending on tissue type. Increases in intestinal iron absorption have been documented in animals with vitamin A deficiency (Roodenburg *et al.*, 1994; Sijtsma *et al.*, 1993). However, vitamin A deficiency has also been shown to cause a 40 - 50% decrease in incorporation of radiolabeled iron into erythrocytes (Mejia *et al.*, 1979; Gardner *et al.*, 1979). These results, in combination with the studies on hepatic iron availability, suggest that vitamin A deficiency associated hepatic iron accumulation prevents bone marrow iron availability for erythropoiesis. This theory is both supported and contradicted in studies published by Roodenburg (Roodenburg *et al.*, 1996; Roodenburg *et al.*, 2000). The first study showed that repletion of vitamin A in deficient rats increased bone iron utilization (Roodenburg *et al.*, 1996), however the latter study showed that erythropoiesis and erythrocyte turnover were not affected by mild vitamin A deficiency (Roodenburg *et al.*, 2000).

Studies have also been conducted to establish the effects of the vitamin A supplementation on iron homeostasis. In humans, radiolabeled iron consumption in combination with two separate forms of supplementation, vitamin A and beta-carotene, enhanced the absorption of non-heme iron (Garcia-Casal *et al.*, 1998). However, many studies use RA supplementation, which acts differently than other vitamin A compounds, due to RA acting as a ligand for retinoic acid receptors (RARs; Kliewer *et al.*, 1992). These RARs and 9-*cis*-retinoic acid receptors (RXR) are nuclear receptors related to the steroid and thyroid hormone receptors, which form heterodimers and act as transcription factors by binding to specific DNA recognition sequences upstream of target responsive genes (Chambon, 1996). Additionally, another receptor has been identified as the RA response

element (RARE; Beato, 1989). This RARE is the binding site for the RAR-beta gene which transcriptionally activates all three RARs (alpha, beta, and gamma).

Due to the complex molecular regulation of RA, administration of RA in specific tissues has produced varying effects on iron homestasis. In neural cells, RA administration induced a 2-fold increase in ferritin-H mRNA after 4 weeks (Vanlandingham and Levenson, 2003). This same study found a similar induction of ferritin in rat brain tissue 24 hours after RA supplementation. Promonocytic cells have exhibited RA mediated decreases in TfR expression (Iturralde *et al.*, 1992) while increased TfR mRNA levels have been exhibited in keratinocytes (Taylor *et al.*, 1985). The effect of RA may be modulated in some cells due to a retinoid-responsive gene (C/EBP) located on certain mRNAs, i.e. myeloid cells for example mRNAs (Chumakov *et al.*, 1997). However, in the next chapter we propose a different model of RA mediation of iron homeostasis. Our group proposes the effects vitamin A has on iron homeostasis are caused by vitamin A interaction with IRPs, consequently altering several proteins involved in iron homeostasis.

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CHAPTER 3 - IRON REGULATORY PROTEINS PLAY A ROLE IN VITAMIN A MODULATION OF IRON HOMEOSTASIS

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ABSTRACT

Vitamin A status has been widely associated with alterations of iron homeostasis. Despite the numerous studies that reiterate the association of these two nutrients, the mechanistic basis of the association has not been well characterized. Recently, iron regulatory proteins (IRPs) have been established as the central regulators of iron homeostasis. Thus, we hypothesize that IRPs play a role in the perturbation of iron homeostasis in vitamin A deficiency and supplementation. Our studies in cultured cells and in rats ($n = 6$) tested this hypothesis by measuring IRP-RNA binding activity along with other iron status indices. In human hepatoma (HepG2) cells we found IRP-RNA binding activity was induced when cells were treated with an iron chelator and slightly repressed when administered retinoic acid (RA). Hence, we measured both iron and vitamin A status indices in rats to determine the role of IRPs in iron deficiency, vitamin A deficiency and a combination of these deficiencies. Acute iron deficiency in rats significantly induced hepatic IRP-RNA binding activity and reduced hepatic ferritin to undetectable amounts. Furthermore, we examined the effect of RA supplementation in these rats. RA supplementation inhibited the increase in IRP-RNA binding activity to a level not significantly different from the control. This inhibition of IRP-RNA binding activity by RA supplementation was correlated with a 34% reduction in transferrin receptor (TfR) abundance and a partial restoration of hepatic ferritin abundance. Our additional studies

utilizing HepG2 cells provided evidence that RA mediates ferritin in a dose-dependant manner. Together, these results indicate that IRPs are involved in vitamin A perturbation of iron homeostasis; a key characterization for understanding this nutrient-nutrient interaction.

INTRODUCTION

Vitamin A and iron deficiencies are major nutritional issues that affect billions of people worldwide (World Health Organization, 2005). Moreover, clinical studies dating back to the 1920's have demonstrated the associations between these nutrients (Koessler *et al.*, 1926).

While iron is essential for nearly all cells, over accumulation of iron can cause toxicity. For this reason, humans have cellular mechanisms in place to control many of the proteins involved in regulating iron homeostasis. Iron homeostasis is regulated through transport (i.e. transferrin), cellular uptake (i.e. transferrin receptors; TfR), and storage (i.e. ferritin) processes. The abundance of these proteins dictates the cellular usage of iron, ensuring proper iron metabolism and homeostasis. Consequently, regulating the production and degradation of these proteins is critical in preventing iron toxicity or deficiency. The main governing proteins which regulate the quantity of the previously listed iron proteins are iron regulatory proteins (IRPs). IRPs bind to hairpin loop structures, iron responsive elements (IREs), located on either the 3'- or 5'- region of mRNA. The binding location regulates the translation of the mRNA message and affects the amount of protein being synthesized (**Figure 3.1,** Eisenstein and Blemings, 1998). If the binding occurs on the 5' region, translation of the mRNA will be blocked, thus inhibiting synthesis of the protein (e.g. ferritin; Barton *et al.*, 1990). However, binding that occurs on 3'-untranslated region

increases the stability of the mRNA message, therefore inducing synthesis of the protein from that particular mRNA code (e.g. TfR; Erlitzki *et al.*, 2002). Because IRPs play a critical role in regulating iron proteins, studies have examined how iron status affects IRP activity. For example, Chen and her colleges established a causal link between iron deficiency and induction of IRP activity (Chen *et al.*, 1998).

Not only has iron deficiency been associated with increases in IRP activity, but has also been documented in the presence of vitamin A deficiency in many observational studies. Both human and animal studies have consistently demonstrated altered iron status indices in vitamin A deficiency (Mejia *et al.*, 1977; Bloem *et al.*, 1989) and altered vitamin A status in iron deficiency (Hodges *et al.*, 1979). These observations have suggested that vitamin A plays a role in perturbing iron homeostasis and vice versa. Furthermore, studies have shown that decreased iron intake in rats can cause accumulation of vitamin A in hepatic tissues (Jang *et al.,* 2000; Strube *et al.*, 2002). However, supplementation of vitamin A, as retinoic acid (RA), in humans has shown to increase both serum iron (Mejia and Chew, 1988) and ferritin (Angeles-Agdeppa *et al.*, 1997), suggesting RA may be modulating iron absorption or cellular export.

Taking together the role of IRPs in iron homeostasis and vitamin A modulation of iron homeostais, we have hypothesized that IRPs play a role in vitamin A modulation of iron status and proteins. Consequently, we designed our studies to test the dependent and independent effects of iron and vitamin A on IRP-RNA binding activity. Our central animal study design was similar to other studies (Rosales *et al.*, 1999; Ameny *et al.,* 2002; Strube *et al.*, 2002); however our study design allowed us to examine acute stages of iron deficiency. This early stage of iron deficiency allowed us to determine how IRP-RNA binding activity

and iron homeostasis are altered in early stages of iron deficiency. Additionally, our studies in hepatic tissue culture provide further insight into the role IRPs play during vitamin A supplementation and varying iron states. We expect these studies to provide insight into a mechanistic link between cellular iron distribution and vitamin A.

MATERIALS AND METHODS

Chemicals

All chemical reagents were obtained from the following: ATRA, Sigma-Aldrich (St. Louis, MO); Calbiochem (La Jolla, CA); cell lines, ATTC (Manassas, VA); ferritin antibody, Boehringer Mannheim (Indianapolis, IN); hemoglobin kit, Wako Diagnostics (Richmond, VA); protease inhibitors and goat anti-rabbit IgG horseradish peroxidase, Southern Biotechnology (Birmingham, IL); Riboprobe System T7 for IRE assay preparation, Promega (Madison, WI); rodent diet components, Harlan Teklad (Madison, WI); transferrin receptor antibody, BD Pharmingen (San Diego, CA); [α-^{32}P] Uridine 5'-triphosphate, PerkinElmer Life and Analytical Sciences (Boston, MA); western blotting detection reagents, Amersham Pharmacia (Piscataway, NJ) and the IRE DNA was kindly provided by Dr. Rick Eisenstiens Lab, University of Wisconsin, Madison (Madison, WI). All other chemicals were analytical grade.

Cell Culture and Study Design

All cell culture experiments used human hepatoma HepG2 cells, which were grown from frozen cultures in our laboratory. Cells were allowed to grow in 150-cm^2 flasks in a humidified incubator with 5% CO_2 and 37°C temperature in media (minimum essential medium) that contained 10% fetal bovine serum, sodium pyruvate (2mM), penicillin (100

units/mL), and streptomycin (0.1 mg/mL). Prior to treatment, cells were split into 75-cm^2 flasks and grown until they were approximately 80% confluent.

Cell culture study 1

For the first study, HepG2 cells were treated with 0.1, 1.0, 10, 50 μM RA dissolved in DMSO or equal volume DMSO (control) and added to the media. Following the 3-day treatment, cells were detached using 5 mL 0.25% trypsin with 1mM EDTA. The cell solution was then washed with Hank's balanced salt solution, centrifuged at 3000 *x g*, followed by aspiration of the solution from the cells. Finally, cells were lysed with the addition of equal volume buffer containing 10 mM HEPES (pH 7.4), 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 50 mM β-glycerophosphate, 5 mM EDTA, 1 mM sodium orthovanadate, 2 mM benzamidine, 100 μg/mL leupeptin and pepstatin, 250 μg/mL soybean trypsin inhibitor, 0.2 mM PMSF, 24 μg/mL *p*-nitroguanidinobenzoate and 0.5% Nonidet P-40. Once the lysis buffer was added to the cells, the mixture was vortexed and iced for approximately 15 minutes. The cell lysate was then centrifuged for 8 minutes at 13,500 rpm (16,000 *x g*) and the supernatant was stored in two portions at -70°C for protein, ferritin, and IRP analysis. The small aliquot of each treatment were frozen separately and measured for protein concentration using a commercial reagent (Coomassie Plus, Pierce) via the Bradford method as described by Bradford (1976). All subsequent analyses used adjusted sample amounts to equalize protein content through all samples.

Cell culture study 2

This cell culture study used HepG2 cells treated with an iron chelator to mimic iron deficiency or an iron source to mimic iron toxicity. Additionally, RA was administered to

half of each group (n =1) to determine the effects of vitamin A supplementation in hepatic tissue with various iron states. Cell treatment groups included: control (DMSO), RA (50 μM RA), desferal (100 μM desferal + DMSO), hemin (20 μM hemin + DMSO), desferal + RA (100 μM desferal + 50μM RA), hemin + RA (20 μM hemin + 50 μM RA). Media and treatments were renewed daily for 3 days. At the end of the study, the same lysis, storage, and analysis procedures, as in the previous cell culture study, were completed.

Animals and Experimental Design

All animal experiments were approved and conducted in accordance with Iowa State University Laboratory Animal Resources Guidelines. Forty-eight male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rats (35-50g) were housed in plastic cages in a locked room that had a twelve-hour light:dark cycle. Animals were given food and water *ad libitum.*

Rats were acclimated to a control diet (as described by Rowling and Schalinske, 2003). Following the acclimation period, rats were randomly assigned to one of two dietary groups (24 rats per group): control or vitamin A-deficient (-A; as shown in **Figure 3.2**). For the first 4 weeks, the rats either consumed the control diet regimen or the same dietary mixture minus the retinyl palmitate. At the fourth week of the study, half of the rats in the control and vitamin A-deficient groups were placed on an iron-deficient diet for the remainder of the study. This made 4 groups of rats (n=12): control, -A, iron-deficient (-Fe) or both vitamin A and iron deficient (-Fe/-A). No pair-feeding was included as other studies have shown no significant differences in the weights of pair-fed rats and rats on deficient diets (Rosales *et al.*, 1999). At the end of week eight, half of each diet group (n=6) was

administered an oral daily dose of RA (30 μmol/kg body weight) or vehicle (corn oil). Following the 7-d treatment period of RA, rats were administered anesthetic (ketamine: xylazine, 90: 10 mg/kg body weight) and sacrificed. Blood samples were collected with heparinized needles via cardiac puncture. Whole blood samples were immediately used for hematocrit determination and the remaining blood was stored at 4°C for subsequent hemoglobin analysis. A 1 gram liver portion was homogenized in 5 volumes IRP buffer containing 10 mM Hepes (pH 7.5), 40 mM potassium chloride, 3 mM magnesium chloride and 0.32 M sucrose with protease inhibitors: 1 mM dithiothreitol, 0.1 mM PMSF and 0.05 mM Leupeptin. Homogenate was centrifuged at 20,000 *x g* for 30 minutes at 4°C and supernatant was stored at –70°C. Liver supernatant was used for protein, hematocrit, hemoglobin determination, ferritin, TfR, and IRP-RNA binding activity and abundance. Liver tissue was wrapped in four separate sections of foil, three 1 gram sections and the remaining tissue in the last section. These were snap-frozen in liquid nitrogen then at –70°C for analysis of liver vitamin A and non-heme iron.

Hematocrit Determination

Capillary tubes were filled with blood collected from cardiac puncture in the rats. These tubes were immediately sealed at one end and stored for less than 2 hours prior to analysis. A hematocrit centrifuge was used to centrifuge the capillary tubes for 5 minutes at 2000 rpm in order to separate the plasma from the red blood cells. Subsequently, the packed red blood cell volume was determined by measurement on a standard scale and expressed as a percentage of the total blood volume collected per sample.

Hemoglobin Determination

Frozen whole blood samples were used to determine hemoglobin concentration, since hemolysis does not have any effect on hemoglobin concentrations. Concentrations were spectrophotometrically determined at 540 nm using a previously established hemoglobin kit (Hemoglobin B Test Wako; Wako Pure Chemical Ind.; Hamaguchi *et al.*, 1992; Oshiro *et al.*, 1982). Briefly, standards were made according to Wako protocol and a standard curve was produced similar to the one provided in the standard operating procedure. Samples were processed by mixing 5 mL of a working solution (1:10 dilution of color reagent and deionized water) with 0.02 mL of sample. Intensities of the samples were established after a 3 minute incubation period at room temperature by reading all samples at 540nm against a reagent blanked spectrophotometer. Concentrations (expressed as g/dL) were determined by dividing the absorbance of the specimen by the absorbance of the standard and multiplying that number by the concentration of the standard solution (15.5g/dL).

Measurement of Liver Vitamin A

Frozen liver samples were analyzed in Dr. Tanumihardjo's laboratory at the University of Wisconsin-Madison on an HPLC, as described by Tanumihardjo *et al.* (1990). Briefly, 1 gram liver samples were ground with ~ 3 times volume sodium sulfate. An internal standard (retinyl buterate) was added to each sample and then the sample was repeatedly extracted with dicholoromethane until the extraction volume equaled 50 mL. A portion of the extraction volume was dried down under argon (5 mL for vitamin A deficient rats and 1 mL for vitamin A sufficient rats), and then reconstituted in 100 μL of 50:50 methanol:ethylene dicholoride. The mixture was then injected into a dual-wavelength

gradient high pressure liquid chromatography (HPLC) system. The two solvents for the HPLC used a ratio of 95:5, acetonitrile to water, containing 10 mM triethylamine (solvent A) and a ratio of 85:10:5, acetonitrile to methanol to dicholoroethane, containing10 mM triethylamine (solvent B). The samples were monitored at 325 and 350 nm and quantified using a retinyl buterate standard to determine retinyl butyrate and other retinyl esters. Finally, calculations converted peak areas to nmol of vitamin A per gram wet weight of liver with adjustment of internal standard amount.

Analysis of Liver Non-heme Iron

The non-heme iron content of the rat livers was measured through a modified method reported by Reddy and Cook (1991). Briefly, 1 gram liver samples were homogenized in 20 mL deionized water for 30 seconds. Only 0.4 mL of the homogenate was used to add to an equal volume 20% TCA/6N HCL and incubated for 20 hrs at 65°C. At 20 hrs, each sample was centrifuged at 3,000 rpm for 15 minutes to remove the precipitated protein. The supernatant was used for the microplate colorimetric assay to in 30 µl duplicates (60 µl for low iron samples). All wells were brought up to 300 µl with working chromogen (1:5:5; stock chromogen, saturated sodium acetate, iron free water). Following a 10 minute incubation period at room temperature, the absorbance was read at 564 nm using a microplate reader. Standards were used to format a standard curve and calculate sample concentrations.

Determination of Ferritin and Transferrin Receptor Abundance

Tissue or cell lysate supernatant samples were organized by treatment groups and ran on separate gels to limit gel to gel variation. The immunoblotting method used was

described previously by our laboratory (Rowling and Schalinske, 2003) and others (Chen *et al.*, 1997; Schalinske & Eisenstein, 1996; Schalinske *et al.*, 1998).

In short summary, a 10-20% SDS-polyacrylamide gel matrix was used to separate the proteins of interest: ferritin and TfR, at 20 and 80 kDa, respectively. Following the separation, the proteins were transferred electrophoretically to nitrocellulose paper and incubated, separately, overnight with a 1:5000 dilution of each primary polyclonal ferritin and TfR antibody. Following the first incubation, each membrane was washed and incubated with a secondary antibody (goat anti-rabbit IgG horseradish peroxidase) for 1 hour at room temperature. Finally, the antibodies attached to the proteins were visualized with chemiluminescence by exposing the membranes to film. SigmaGel software (SPSS, Chicago, IL) was used for the densitometric analysis. Samples densities were subtracted from their background and immunoblots were compared by separating out variation between immunoblots.

Analysis of Iron Regulatory Protein Activity and Abundance

IRP binding activity was measured by IRP-IRE binding ability, utilizing an electrophoretic mobility shift assay (Barton *et al.*, 1999; Eisenstein *et al.*, 1993). The L-ferritin plasmid vector containing an IRE was digested with a nuclease that cuts immediately after the IRE hairpin loop structure. A ^{32}P-UTP labeled RNA was made by combining the RNA and T7 RNA polymerase and purifying the compound through a 10% acrylamide 8 M urea gel. The half life of the radioactivity used was 14-d and the specific radioactivity of the synthesized RNA was 6,000-7,000 dpm/fmol. The RNA IRE-IRP binding assay was completed by separating the previously active, or bound, and free IRE with non-denaturing

gel electrophoresis. A liquid scintillation counter was used to measure the radioactive IRE-IRP complex to establish active IRP quantities. To establish the total endogenous IRP, prior to repeating the same assay, samples were treated with 3% 2-mercaptoethanol. This treatment allowed measurement of total IRP by converting all inactive IRPs to the active RNA-binding form.

Statistical Analysis

All statistical analyses were completed using SigmaStat software (SPSS, Chicago, IL). The treatment groups were subjected to a one-, two- or three-way ANOVA, depending on the data. The means were compared using Fisher's Least Significant Difference analysis method when the ANOVA was significant ($p < 0.05$).

RESULTS

RA increases intracellular ferritin in a dose-dependant manner in human hepatoma cells. Low doses of RA (0.1, 1, 10μM) in HepG2 cells caused a slight increase in ferritin, with the highest increase of these treatments producing a 1.5-fold increase from the control group (**Figure 3.3**). In contrast, the highest dose of RA (50μM) induced ferritin nearly 3-fold. The RA mediated induction of ferritin did not appear to be associated with changes in IRP-RNA binding activity as these cells showed no significant changes in this activity **(Figure 3.4)**.

RA supplementation effects IRP-RNA binding activity in iron sufficient and deficient hepatoma cells. Iron chelation in human hepatoma cells induced IRP-RNA binding activity 27% while iron administration to the same cell line failed to alter IRP-RNA binding activity (**Figure 3.5**). RA administration in the hepatoma cells decreased the IRP-

RNA binding activity 7%, as a percent of total abundance, in the iron chelated cells (i.e. desferal) and increased the activity 1 - 3% in both control and hemin administered cells.

Iron status indices reflected acute stages of iron deficiency in rats consuming iron deficient diets. Acute iron deficiency resulted from inconsistent changes in various iron indices. Experimental analysis showed that the rats consuming an iron deficient diet exhibited a 50% decrease in liver non-heme iron ($p<0.001$), a 10% decrease in hematocrit ($p=0.008$) and no significant alteration of hemoglobin levels ($p=0.191$; **Table 3.1**).

Vitamin A deficiency in rats decreases hepatic vitamin A, while iron deficient rats accumulate hepatic vitamin A. The rats on a vitamin A deficient diet exhibited nearly undetectable amounts of vitamin A in hepatic tissue (**Table 3.2**). The hepatic vitamin A of the vitamin A deficient rats was significantly lower than the control rats ($p<0.001$). Furthermore, rats on an iron deficient diet exhibited a 66% increase in hepatic vitamin A (Table 3.2).

TfR abundance is inhibited by RA supplementation. TfR abundance decreased by 34%, regardless of dietary treatment. Despite the insignificant effect of dietary treatment on TfR values ($p=1.114$), pooling groups into control and RA supplemented categories allowed significance to be established ($p=0.01$; **Figure 3.6**).

Iron deficiency depleted iron stored as ferritin while RA supplementation induces ferritin abundance. Rats that consumed an iron deficient diet for 5 weeks became depleted in hepatic ferritin (**Figure 3.7**). This condition was greatly altered by RA supplementation. RA increased in the hepatic concentrations of ferritin 1.7-fold in control

rats, 1.2-fold individually in vitamin A deficient and iron deficient rats and 2-fold in the rats consuming the combination deficient diet (Figure 3.7).

Elevated IRP-RNA binding activity in rats consuming iron deficient diets was decreased by RA supplementation. Iron deficiency, with (p=0.004) or without vitamin A deficiency (p=0.012), significantly induced IRP-RNA binding activity (**Figure 3.8**). Although RA supplementation did not significantly alter IRP-RNA binding activity in the control (p=0.757) or vitamin A deficient groups (p=0.906), inductions of IRP-RNA binding activity were eliminated in the rats consuming iron deficient diets through RA supplementation (p=0.058 for the –Fe group and 0.172 for the –Fe/-A group).

DISCUSSION

Establishing a mechanistic basis of the relationship between vitamin A deficiency and iron deficiency is vital due to the extensiveness of these deficiencies worldwide. Many research groups have studied the effects of vitamin A status on indices of iron status (Roodenburg *et al.*, 2000; Strube *et al.*, 2002; Kelleher and Lonnerdal, 2005). Some research has even examined other outcomes to determine effects of vitamin A alteration of iron homeostasis e.g. neuronal differentiation (Vanlandingham and Levenson, 2003). Even though these studies contribute to the understanding of how these nutrients are associated and the outcomes of their interactions, they do not establish the mechanistic link between this association. Therefore, we designed our studies to shed light on what we believe is the major connecting unit in the relationship between vitamin A and iron: IRPs.

Due to the governing role IRPs in iron homeostasis, our group measured IRP-RNA binding activity in the animal study to determine its role in vitamin A modulation of iron

homeostasis. Prior to the animal study, we examined the effects of iron deficiency and supplementation on IRP-RNA binding activity in hepatic cultured cells. Our hepatoma cells reflected the different effects of iron chelation and administration on IRP-RNA-binding activity with a 27% induction and no alteration, respectively. Our results were similar to those produced by Oxele *et al.* when they measured IRP-mediated indices, i.e. m-acon (1999; Figure 3.8).

Our animal study reflects the effects of RA supplementation in rats with iron or vitamin A compromised status alone or in combination. Other studies have assessed the outcomes of this interaction, many of which have been documented and are nicely compiled in a paper by Semba and Bloem (2002). However, we have supplied additional endpoints to provide ample data for accurate insight into the mechanistic basis for how iron homeostasis is regulated by RA. Stages of iron deficiency have varied in vitamin A deficiency studies. Roodenburg *et al.* published three studies reporting reduced hemoglobin and hematocrit in vitamin A deficiency (Roodenburg *et al.*, 1994, 1996a, 1996b). However, one of her more recent studies (Roodenburg *et al.*, 2000), along with a study from 1979 (Hodges *et al.*, 1979), found no changes in hemoglobin or hematocrit due to vitamin A deficiency. Our study agrees with the latter findings. Rats on vitamin A deficient diets in our study did not exhibit significantly altered iron status indices. Although, we were able to produce iron indices reflective of acute stage iron deficiency in rats consuming an iron deficient diet. This state was established by some iron indices indicating iron deficiency, i.e. liver non-heme iron and hematocrit, while one did not, i.e. hemoglobin (Table 3.1).

Vitamin A analysis in our study confirmed earlier published data that iron deficiency results in hepatic retinol accumulation (Jang *et al.*, 2000; Strube *et al.*, 2002). Our study

produced a significant 60% - 71% increase in liver vitamin A between the control and iron deficient rats. These data suggest that iron deficiency promotes the sequestration of vitamin A in the liver.

Previous animal studies on iron deficiency have exhibited an induction of hepatic TfR abundance (Ponka, 1997) and reduction of hepatic ferritin (Chen *et al.,* 1998). However, studies in other cell types, e.g. erythroblast, which have shown induced TfR abundance in iron chelated cells, have failed to find reductions of TfR abundance in cells containing sufficient iron (Abe *et al.,* 1992). Our results did not produce a significant reduction of TfR abundance in rats consuming an iron deficient diet; however when we pooled dietary groups, we did find a significant 34% reduction in TfR abundance in response to RA administration (Figure 3.3).

In regards to ferritin abundance, our study supported many studies where both iron deficient rats exhibited almost nonexistent ferritin abundance (Roodenburg *et al.*, 1994; Chen *et al.*, 1998; Roodenburg *et al.*, 2000; Strube *et al.*, 2002; Kelleher and Lonnerdal, 2005) and a few studies where RA induced ferritin (Roodenburg *et al.*, 2000; Strube *et al.*, 2002; Kelleher and Lonnerdal, 2005). Preliminary data from ferritin and TfR abundance in K562 and intestinal Caco-2 cells indicate that these cells are not as responsive to RA supplementation as hepatoma HepG2 cells in iron deficient and sufficient states (data not shown). Our animal data, reflected in Figure 3.5, exhibits partial restoration of ferritin due to RA treatment. Additionally, our cell culture data provides evidence that the RA induction seen in the hepatic tissue of rats is a dose-dependant mechanism (Figure 3.3). However, the dose-dependant induction of ferritin in the latter cell culture study, did not illicit any

perturbations in IRP abundance, suggesting RA may be altering IRP-RNA binding activity and ferritin in a posttranslational manner suggested in other studies (Schalinske *et al.*, 1998).

Together, our studies provide a crucial look into the mechanistic relationship between vitamin A and iron homeostasis, namely IRPs. To our knowledge, no one has examined IRP's role in the interaction of these two nutrients. IRP's have the potential to be crucial to this relationship seeing as they are able to regulate the production of the proteins that dictate iron homeostasis (Eisenstein, 2000). We have provided evidence that IRPs do play a role in RA alterations of iron homeostasis in iron deficiency. Taken together, these results not only support the theory that vitamin A supplementation is necessary in iron supplementation programs, but also indicates that vitamin A supplementation results in iron accumulation in hepatic tissue. We are the first to illustrate the potential role IRPs play in perturbation of iron homeostasis by vitamin A. Further research is necessary to establish if RA alteration of iron status is dependent of IRP alterations or if the reduction in IRP's is an outcome of the increase in intracellular iron.

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FIGURE LEGENDS

Figure 3.1

Post-transcriptional regulation of ferritin and transferrin receptor (TfR) by iron regulatory proteins (IRPs, Eisenstein and Blemings, 1998). IRP1 and IRP2 are both depicted in the diagram to exhibit the conservation of IRP1 in the inactive form (aconitase; A-con) and the degradation of IRP2. In a low iron state, active IRP1 and IRP2 bind to iron response elements (IREs), depicted as hairpin loop structures on either the 5' region of ferritin or the 3'-untranslated region of TfR mRNA. The binding location on relative mRNAs causes inhibition of ferritin translation and the stabilization of the TfR mRNA message, thereby increasing translation. Thus, a high iron state de-activates IRPs, thereby inhibiting IRP-IRE binding. The un-bound IREs allow activation of ferritin translation while inhibiting TfR synthesis.

Figure 3.2

Diagram of animal study design. Fourty-eight male Sprague-Dawley rats were randomly assigned to one of two dietary groups (24 rats per group): control or vitamin A-deficient (-A). For the first 4 weeks, the rats either consumed vitamin A deficient the control diet regimen or the same dietary mixture minus the retinyl palmitate. At the fourth week of the study, half of the rats in the control and vitamin A-deficient groups were placed on an iron-deficient diet for the remainder of the study. This made 4 groups of rats (n=12): control, -A, iron-deficient (-Fe) or both vitamin A and iron deficient (-Fe/-A). At the end of week eight, half of each diet group (n=6) was administered an oral daily dose of RA (30 μmol/kg body weight) or vehicle (corn oil). Following the 7-d treatment period of RA, the rats were sacrificed.

Figure 3.3

Retinoic acid (RA) mediation of ferritin abundance in human hepatoma cells. Human hepatic carcinoma cells were treated with 0, 0.1, 1, 10 and 50 μM RA in DMSO. Following a 3-day incubation period cells were collected and lysed. Ferritin abundance was determined via the western blotting method, described in the materials and methods section. A representative immunoblot is shown above the bar graph. Vertical bars represent percent abundance of treatment groups as compared to the control group. Despite the inability to test for statistical significance (due to $n = 1$), RA visibly induced ferritin abundance in a dose dependant manner up to 285%.

Figure 3.4

Regulation of iron regulatory protein (IRP) -RNA binding activity in hepatoma cells treated with varying doses of retinoic acid (RA). The human hepatoma cells, described in figure 3.2, were used to determine IRP-RNA binding activity via the electrophoretic mobility shift assay, as described in the "Materials and Methods" section. The blot shown is representative of the autoradiograms used for the bar graph ($n = 1$). Vertical bars are expressed as means.

Figure 3.5

Retinoic acid (RA) modulation of iron regulatory protein (IRP) –RNA binding activity and abundance in iron deficient or sufficient hepatoma cells. Human hepatic carcinoma cells were treated with an iron chelator (desferal) or an iron source (hemin) for 3 days. Half of each group was administered 20 μM RA during this period ($n = 1$). Following the treatment period, cells were lysed and measured for IRP-RNA binding activity and abundance via the electrophoretic mobility shift assay, as described in the "Materials and Methods" section.

Bars represent mean quantified amount of IRP-RNA binding activity, as a percent of total abundance.

Figure 3.6

Transferrin receptor (TfR) abundance is significantly inhibited in rats supplemented retinoic acid (RA). Rats consumed control, vitamin A deficient (-A), iron deficient (-Fe), or vitamin A and iron deficient (-Fe/-A) diets for 8 weeks. Following the 8 week dietary treatments, half of the rats from each group were supplemented daily with RA (30 μmol/kg body weight) or corn oil for 7 days. Hepatic tissues from these rats were used to determine TfR abundance through immunoblotting procedures, as described in the "Experimental Procedures" section. All dietary groups were pooled into those rats that were supplemented with RA and those that were not (n = 24). Values are expressed as a mean ± S.E.M. Statistical significance is denoted with an asterisk, p=0.01.

Figure 3.7

Modulation of ferritin abundance with iron deficiency and retinoic acid (RA) supplementation. Liver samples were removed from rats described in Figure 3.5. Ferritin abundance was determined by western blotting, as described under "Experimental Procedures". A monoclonal ferritin antibody was used for the western blot analysis, a representative immunoblot is shown. Iron deficient diets (-Fe or -Fe/-A) visibly reduced ferritin abundance, which is partially restored via RA supplementation in those rats, the control rats and the vitamin A deficient (-A) rats (n = 6).

Figure 3.8

Retinoic acid (RA) inhibits iron or vitamin A deficiency mediated induction of iron regulatory protein (IRP) -RNA binding activity in rats. The same rats as described in

figure 3.5 were used. However, when rats were sacrificed, liver homogenate for IRP-RNA binding activity and abundance was used. IRP-RNA binding activity and abundance was determined through an electrophoretic mobility shift assay described in the "Materials and Methods" section. A representative autoradiogram is shown above the graph depicting quantitative data that is expressed as a mean ± S.E.M. (n = 6). Different letters above the bars, representing the quantitative measurement of the IRP-RNA binding activity assay, denoting statistical significance, $p<0.05$ (-Fe, $p=0.012$; –Fe/-A, $p=0.004$).

TABLES AND FIGURES

Table 3.1

Iron indices hematocrit, hemoglobin, and liver non-heme iron from rats treated with all-*trans*-retinoic acid (RA) consuming control, vitamin A deficient (-A), iron deficient (-Fe), or iron and vitamin A deficient diet (-Fe/-A). Values are expressed as mean ± S.E.M. ($n = 6$). Statistical significance is denoted as different letter superscripts, $p < 0.05$.

	Control	-A	-Fe	-Fe/-A
Hematocrit (% of whole blood)				
-RA	42.3 ± 1.1^{a}	$41.8 \pm 1.2^{a,b}$	$38.7 \pm 0.9^{b,c}$	40.2 ± 0.9^{c}
+RA	42.5 ± 0.5^{a}	$41.3 \pm 1.9^{a,b}$	$38.3 \pm 1.6^{b,c}$	39.3 ± 0.5^{c}
Hemoglobin (g/dL)				
-RA	14.4 ± 1.0	11.5 ± 0.8	13.7 ± 0.8	14.8 ± 0.8
+RA	13.7 ± 1.3	15.3 ± 1.4	12.7 ± 1.0	13.7 ± 0.7
Liver non-heme iron (µg/g)				
-RA	1079.2 ± 47.8^{a}	1240.4 ± 111.4^{a}	537.9 ± 47.1^{b}	429.2 ± 66.2^{b}
+RA	1175.3 ± 67.0^{a}	1202.4 ± 72.0^{a}	527.5 ± 68.0^{b}	551.9 ± 50.6^{b}

Table 3.2

Liver concentration of vitamin A from rats treated with all-*trans*-retinoic acid (RA) consuming control, vitamin A deficient (-A), iron deficient (-Fe), or iron and vitamin A deficient diet (-Fe/-A). Values are expressed as mean ± S.E.M. ($n = 6$). Statistical significance is denoted as different letter superscripts in each indice group, $p < 0.05$.

Liver vitamin A (nmol/g liver)				
	Control	**-A**	**-Fe**	**-Fe/-A**
-RA	198 ± 37^{b}	0.6 ± 0.3^{c}	327 ± 23^{a}	0.2 ± 0.1^{c}
+RA	219 ± 25^{b}	0.5 ± 0.4^{c}	307 ± 49^{a}	0.6 ± 0.2^{c}

Figure 3.1

Post-transcriptional regulation of ferritin and transferrin receptor (TfR) by iron regulatory proteins (IRPs, Eisenstein and Blemmings, 1998). Abbreviations: Cytosolic-aconitase (c-acon), iron regulatory protein 1 (IRP1) and 2 (IRP2), iron response element (IRE) and transferrin receptor (TfR).

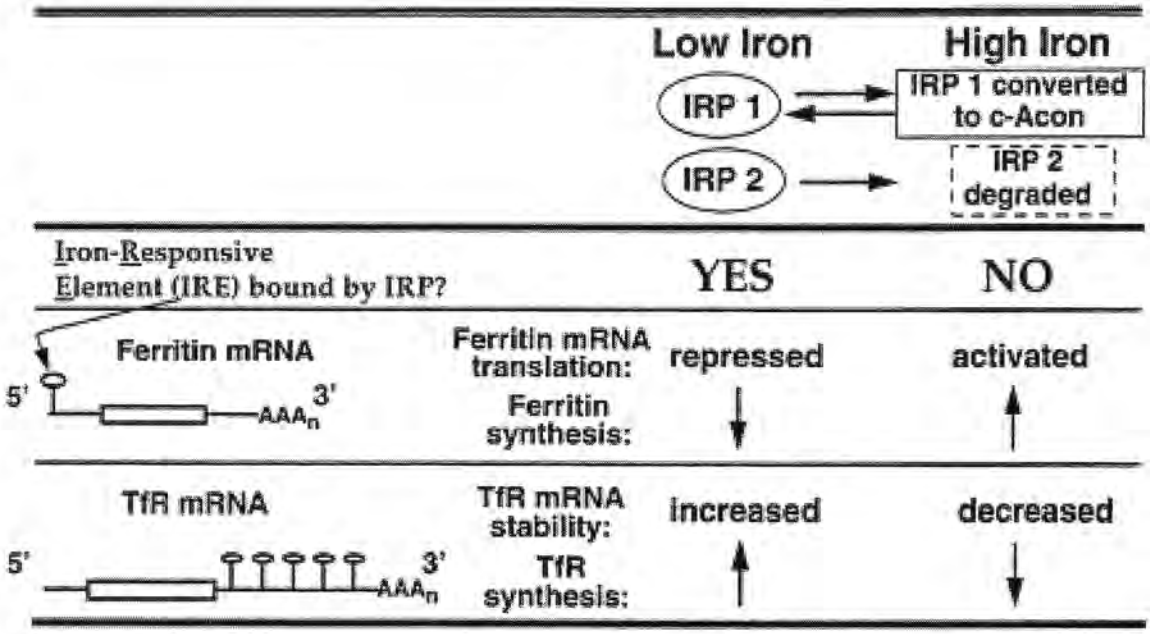


Figure 3.2

Diagram of animal study design.

Rat diet abbreviations: iron deficient (-Fe), vitamin A deficient (-A) and vitamin A deficient plus iron deficient (-Fe/-A).

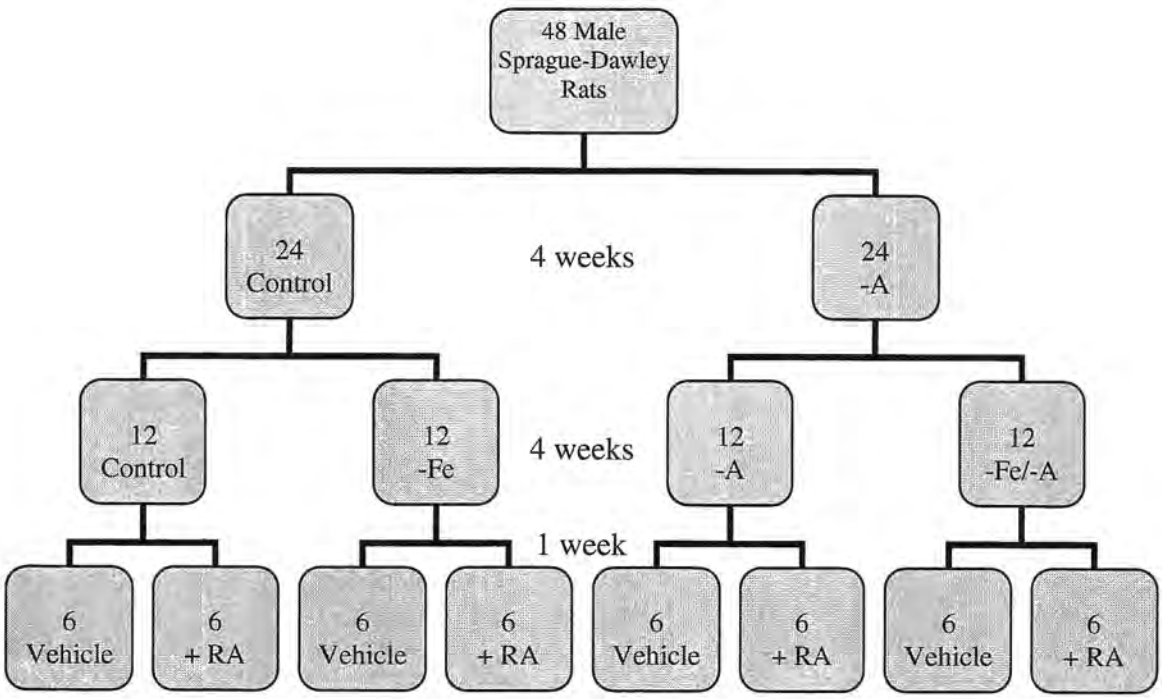
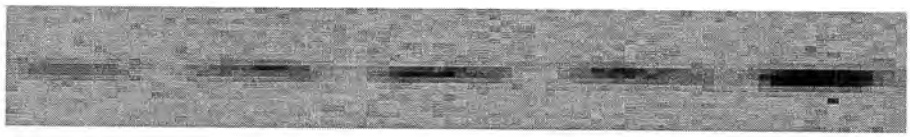


Figure 3.3



RA: 0 0.1 1 10 50

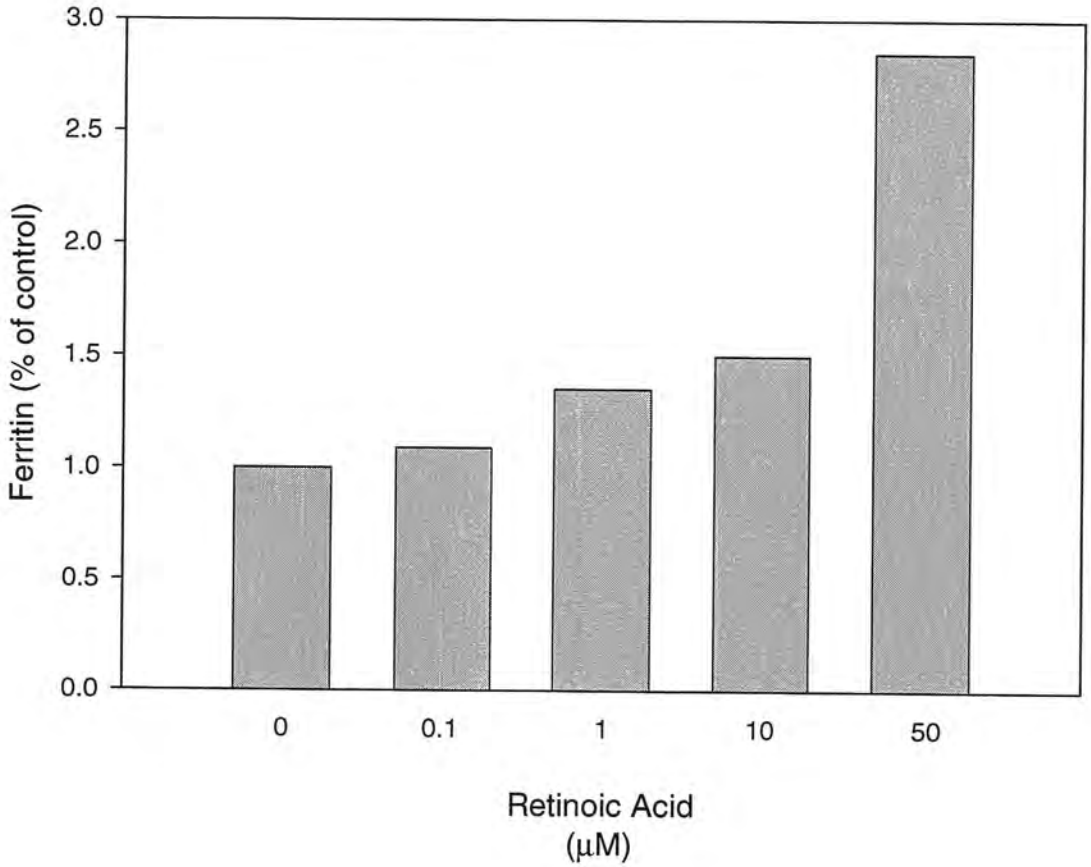
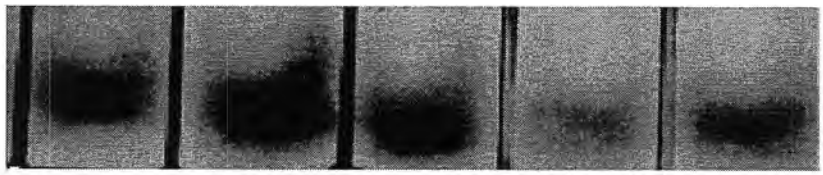


Figure 3.4



RA (μM): 0 0.1 1 10 50

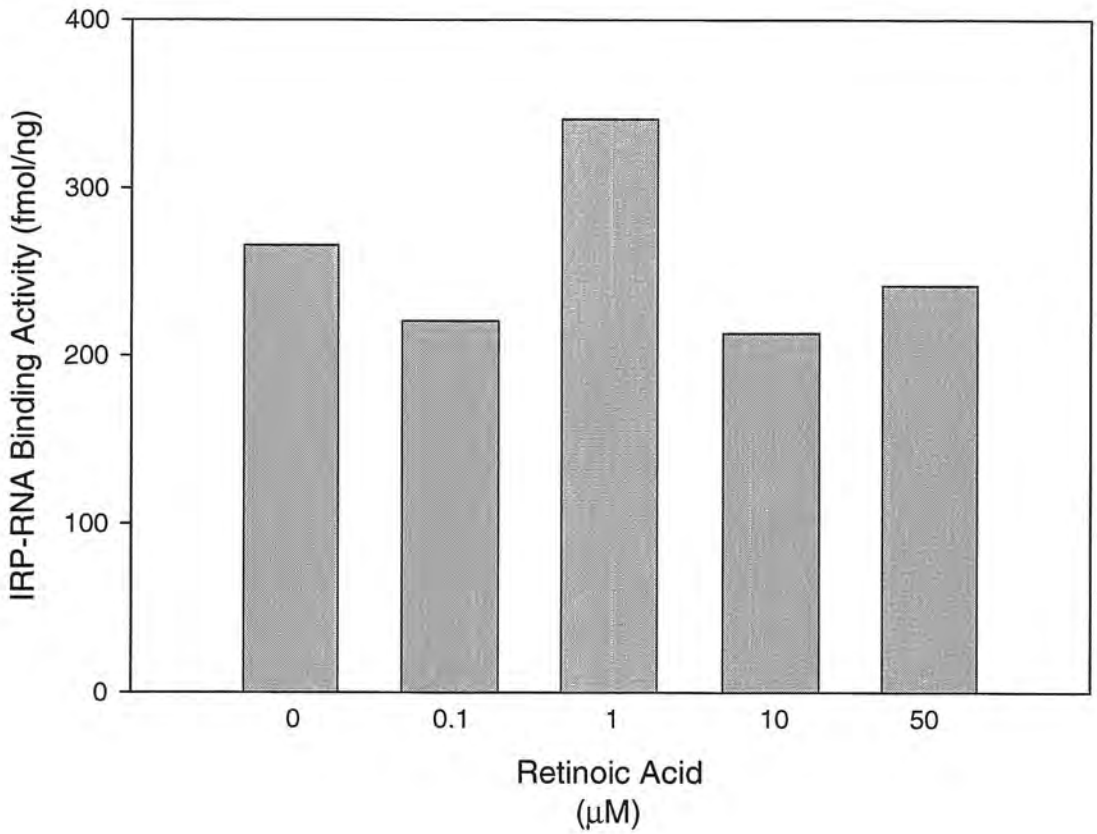


Figure 3.5

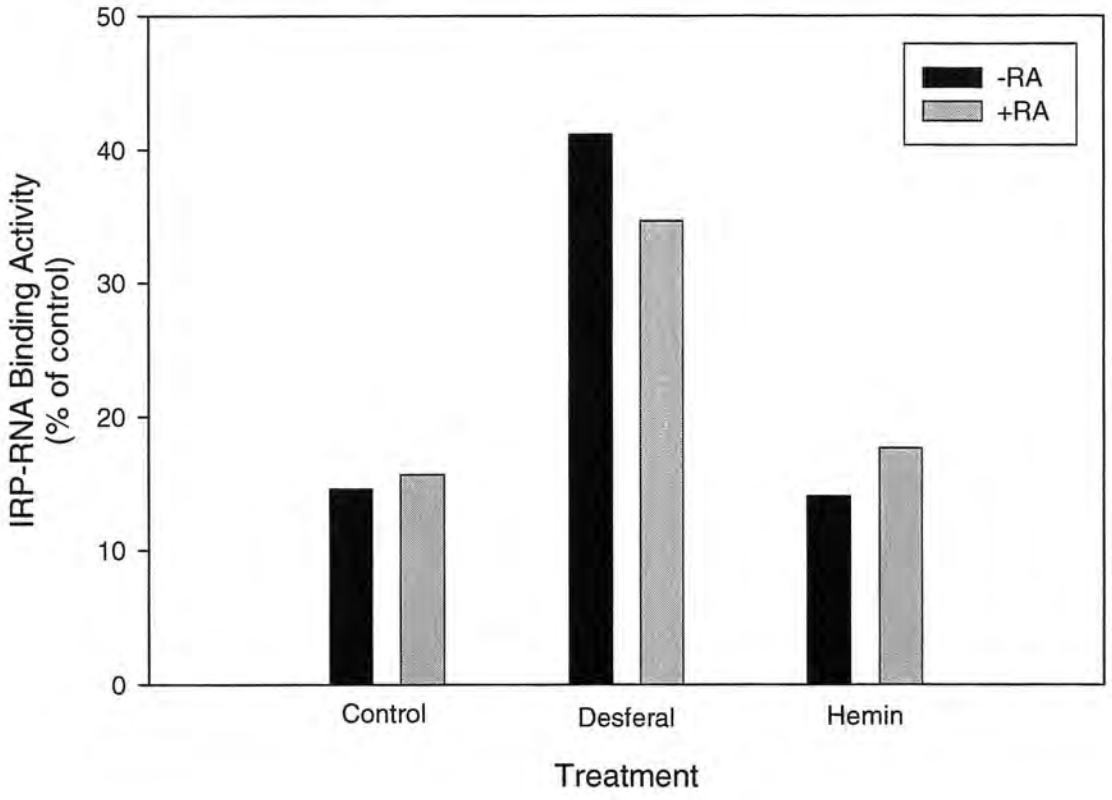

50
40
30
20
10
0
IRP-RNA Binding Activity
(% of control)
-RA
+RA
Control
Desferal
Hemin
Treatment

Figure 3.6

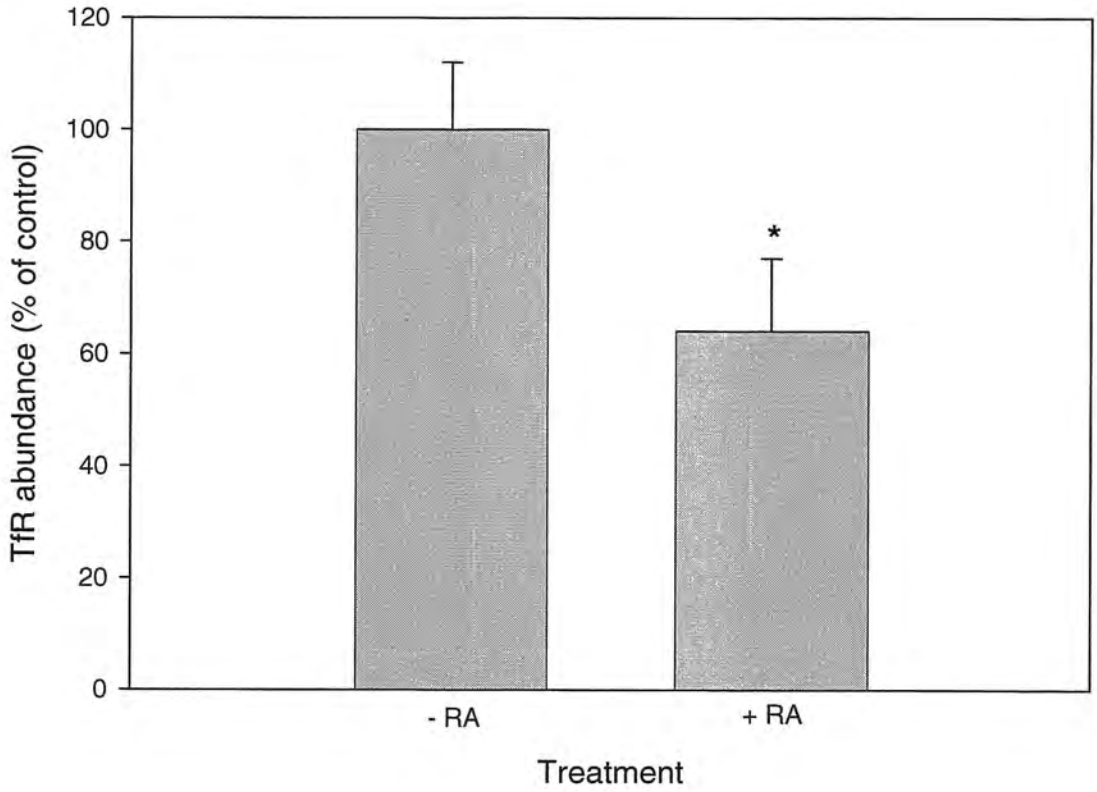


Figure 3.7

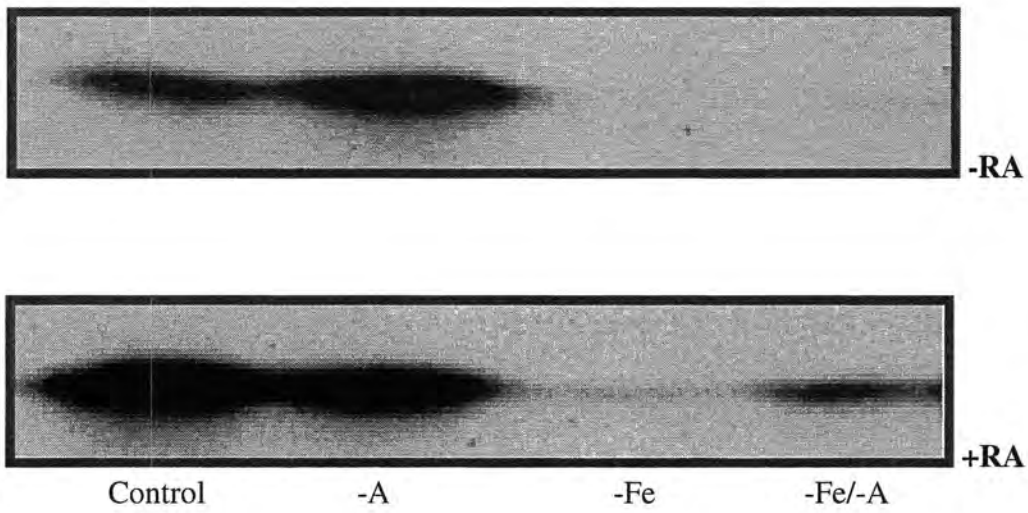
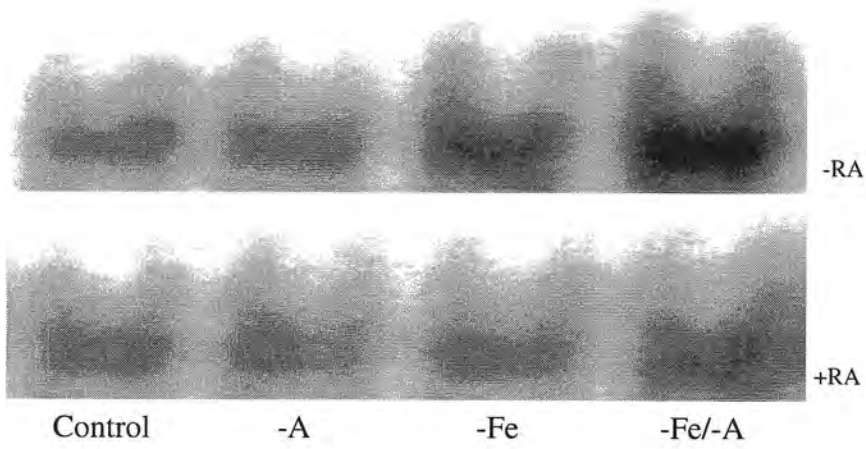
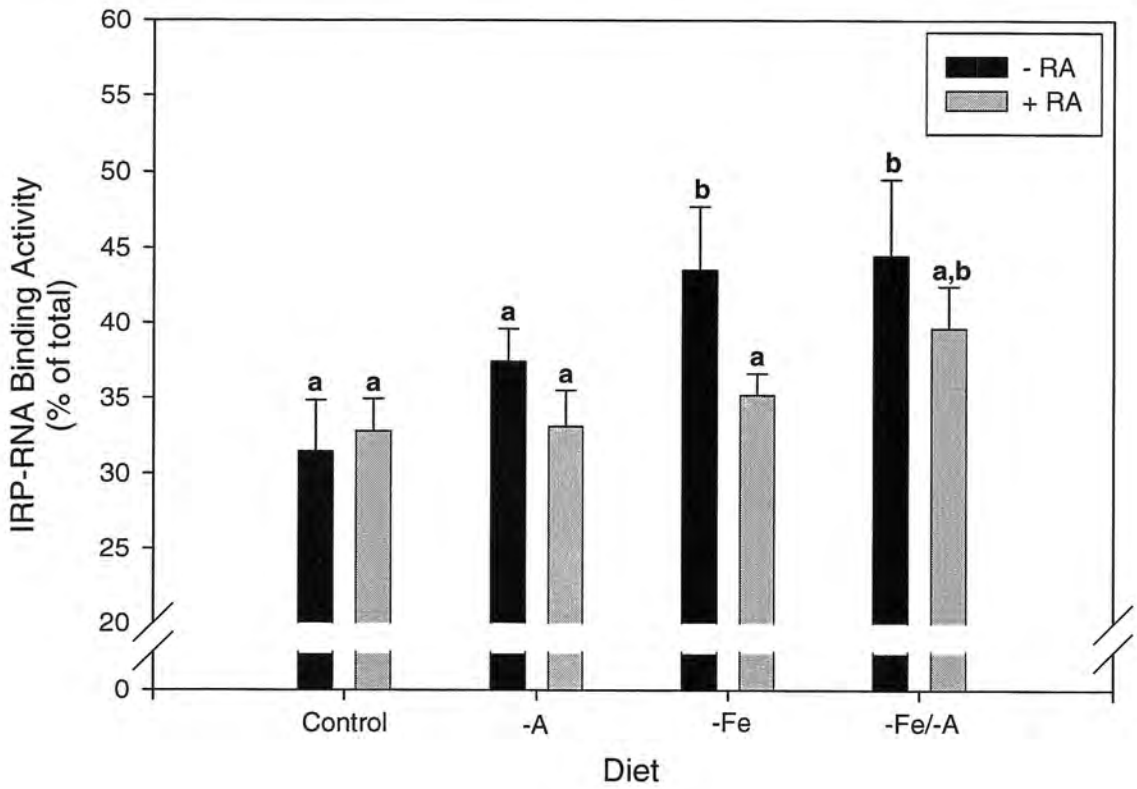


Figure 3.8


-RA
+RA
Control
-A
-Fe
-Fe/-A


IRP-RNA Binding Activity
(% of total)
60
55
50
45
40
35
30
25
20
0
- RA
+ RA
a
a
a
a
b
a
b
a,b
Control
-A
-Fe
-Fe/-A
Diet

CHAPTER 4 – GENERAL CONCLUSIONS

SUMMARY AND CONCLUSIONS

Vitamin A and iron deficiencies prove to be worldwide diseases that affect millions. The well recognized but complex association between these two nutrients has only recently begun to be elucidated. Discovery of several new proteins involved in iron homeostasis has shed light into the mechanistic control of iron homeostasis. Although, several of these proteins have yet to be concisely characterized and studied in various stages of iron deficiency.

As stated several times throughout this thesis, maintenance of iron homeostasis depends on orchestration of all the proteins described in chapter 2. A keystone of iron homeostasis has been established as iron regulatory proteins (IRP). IRPs have been shown to regulate several of the proteins involved in iron homeostasis through posttranscriptional and translational mechanisms. Moreover, IRP activity has been shown to be activated during low iron status and repressed in states of iron abundance (Barton *et al.*, 1990).

Our theory that modulation of vitamin A status would perturb iron homeostasis was examined by our preliminary studies in cell culture and a final animal study. Through examining the interaction of retinoic acid (RA) and iron homeostasis in human hepatoma HepG2 cells, we were able to provided evidence that RA modulates ferritin in a dose-dependant manner, as depicted in other studies (Roodenburg *et al.*, 1994; Roodenburg *et al.*, 2000; Strube *et al.*, 2002; Kelleher and Lonnerdal, 2005). However, the IRP-RNA binding activity data in our first cell culture study did not suggest a significant contribution of IRPs in the alterations of ferritin by RA. In contrast, our second study with HepG2 cells provided

evidence that IRP-RNA binding activity is altered in both iron supplementation and deficiency and RA administration (Figure 3.4). Similar results were seen in our animal model.

In a rat model, the IRP-RNA binding activity was found to be modulated by RA. RA affected IRP-RNA binding activity in a manner that resulted in complete elimination of significant induction of IRP activity from dietary deficiency of vitamin A and iron. Additionally, partial restoration of ferritin abundance provided further evidence that IRPs play a significant role in RA-mediated alteration of iron homeostasis. An advantage of our study is the acute stage of iron deficiency in the rats, allowing our research group and the readers to see what effects RA has early on in iron deficiency. A final significant finding of our study was accumulation of retinol in hepatic tissues of the rats consuming an iron deficient diet, a finding which has been previously documented (Jang *et al.,* 2000; Strube *et al.*, 2002).

Taken together, these results suggest that vitamin A, as RA, results in iron accumulation by the liver. The opposing side of these results, vitamin A deficiency, reflects the significant induction of IRP-RNA binding activity when in combination with iron deficiency. Whether vitamin A deficiency exacerbates the increase in IRP RNA-binding activity to decrease ferritin abundance or whether the inhibition of ferritin abundance by RA induces IRP activity. Specific characterization of this question will require further research. The conclusion will have implications for the millions of people suffering from either iron or vitamin A deficiency. Additionally, the results from RA supplementation have implications for people consuming RA, e.g. acne medication.

RECOMMENDATIONS FOR FUTURE RESEARCH

Although the associations between vitamin A and iron have been distinctly described, future research needs to fully characterize the role of IRPs and other mechanistic processes. Studies should aim to distinguish the role of IRPs in vitamin A alteration of iron homeostasis in tissues other than hepatic (such as intestinal and erythroid). The studies in these tissues, along with hepatic, should be designed to provide a more precise insight into whether ferritin is being altered by vitamin A, which in turn alters IRP, or if vitamin A is perturbing IRPs which in turn regulates abundance of iron proteins such as ferritin and TfR, i.e. time course studies. Moreover, studies should be designed to reflect the relative contribution of IRP1 and IRP2 while incorporating newly elucidated proteins that affect iron homeostasis (DMT1, hepcidin, ferroportin, etc.).

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